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(54) **ANTIGEN-BASED HETEROPOLYMERS FOR TREATING AUTOIMMUNE DISEASES**

ANTIGEN-BESIERENDE HETEROPOLYMERE ZUR BEHANDLUNG VON AUTOIMMUNKRANKHEITEN MITTELS DIESEN

HETEROPOLYMERES A BASE D'ANTIGENE POUR LE TRAITEMENT DE MALADIES AUTOIMMUNES

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(56) References cited:
WO-A-92/05801

- **DATABASE MEDLINE [Online] US NATIONAL LIBRARY OF MEDICINE (NLM), BETHESDA, MD, US TAYLOR R P ET AL: "Complement-opsonized IgG antibody / dsDNA immune complexes bind to CR1 clusters on isolated human erythrocytes." retrieved from STN Database accession no. 92006299 XP002130057 & CLINICAL IMMUNOLOGY AND IMMUNOPATHOLOGY, (1991 NOV) 61 (2 PT 1) 143-60. ,**
- **DATABASE MEDLINE [Online] US NATIONAL LIBRARY OF MEDICINE (NLM), BETHESDA, MD, US FERGUSON P J ET AL: "Antigen-based heteropolymers. A potential therapy for binding and clearing autoantibodies via erythrocyte CR1." retrieved from STN Database accession no. 95151047 XP002130058 & ARTHRITIS AND RHEUMATISM, (1995 FEB) 38 (2) 190-200. ,**
- **W. PAUL, "FUNDAMENTAL IMMUNOLOGY", 3rd. Ed., Published 1993, by RAVEN PRESS (NEW YORK), pages 917, 926-927.**

EP 0 743 856 B1

- JOURNAL OF IMMUNOLOGY, Vol. 148, No. 8, issued 15 April 1992, TAYLOR et al., "In Vivo Binding and Clearance of Circulating Antigen by Bispecific Heteropolymer-Mediated Binding to Primate Erythrocyte Complement Receptor", apges 2462-2468.
- JOURNAL OF CLINICAL INVESTIGATION, Vol. 84, issued September 1989, KIMBERLY et al., "In Vivo handling of Soluble Complement Fixing Ab/dsDNA Immune Complexes in Chimpanzees", pages 962-970.
- JOURNAL OF IMMUNOLOGICAL METHODS, Vol. 132, issued 1990, EMLEN et al., "A New Elisa for the Detection of Double-Stranded DNA Antibodies", pages 91-101.
- BLOOD, Vol. 57, No. 1, issued 1981, MANNUCCI et al., "Precipitating Antibodies to Factor VIII/Von Willebrand factor in Von Willebrand's Disease: Effects on Replacement Therapy", pages 25-31.
- JOURNAL OF NEUROIMMUNOLOGY, Vol. 19, No. 3, issued 1988, BROWN et al., "Clonotypic Analysis of the Antibody Response to the Acetylcholine Receptor in Experimental Autoimmune Myasthenia Gravis", pages 205-222.
- JOURNAL OF NEUROIMMUNOLOGY, Vol. 14, No. 2, issued 1987, MOREL et al., "Increased Binding of Anti-Acetylcholine Receptor Antibodies to Thymic Antigen in Patients with Myasthenia Gravis and Other Autoimmune Diseases Compared to Those with Myasthenia Gravis Alone", pages 221-226.
- CLINICAL AND EXPERIMENTAL IMMUNOLOGY, Vol. 75, No. 2, issued 1989, SUTJITA et al., "A Common Anti-Cardiolipin Antibody Idiotype in Autoimmune Disease Identification Using a Mouse Monoclonal Antibody Directed Against a Naturally-Occuring Anti-Phospolipid Antibody", pages 211-216.
- THE JOURNAL OF INFECTIOUS DISEASES, Vol. 157, No. 1, issued January 1988, HARRIS et al., "Use of an Enzyme-Linked Immunosorbent Assay and of Inhibition Studies to Distinguish Between Antibodies to Cardiolipin from Patients with Syphilis or Autoimmune Disorders", pages 23-31.
- JOURNAL OF IMMUNOLOGICAL METHODS, Vol. 133, isssued 1990, MEILOF et al., "The Detection of Anti-Ro/SS-A and Anti-La/SS-B Antibodies: A Comparison of Counterimmunoelectrophoresis with Immunoblot, Elisa, and RNA-Precipitation Assays", pages 215-226.
- ARCHIVES OF DERMATOLOGY, Vol. 123, No. 3, issued 1987, FALANGA et al., "Antinuclear and Anti-Single-Stranded DNA Antibodies in Morphea and Generalized Morphea", pages 350-353.

Description

Technical Field

[0001] The present invention relates to antigen-based heteropolymers specific for both a specific receptor site on a primate erythrocyte and a target pathogenic autoantibody. The present invention further relates to the use of a composition comprising these antigen-based heteropolymers for the preparation of a medicament for treating autoimmune diseases.

Background Art

[0002] Circulating autoantibodies are responsible for much of the pathogenesis associated with a number of autoimmune diseases including, but not limited to, systemic lupus erythematosus (SLE), autoimmune myocarditis, immune complex mediated kidney disease, rheumatoid arthritis, myasthenia gravis, autoimmune anemias, Sjogren's Syndrome, idiopathic thrombocytopenic purpura, various forms of vasculitis, and at least some of the cellular cytotoxicity accompanying Acquired Immune Deficiency Syndrome (AIDS).

[0003] Efforts to treat autoantibody-mediated disorders have been only partially successful. Many of the past developed therapies have been based on the use of general immunosuppressive measures, through drugs or therapeutic monoclonal antibodies designed to completely abrogate antibody production. However, to date, a successful treatment has not been designed which targets the specific autoantibodies.

[0004] Plasmapheresis, which is designed to remove all the circulating antibodies, has been attempted, but only with limited success. In recent years, a variety of extracorporeal immunoabsorption procedures have been attempted. These procedures are more specific, and attempt to remove only the pathogenic autoantibodies. This involves allowing the blood or plasma to flow over matrices outside the body which contain the autoantigen which is the natural target of the autoantibodies. These methods are slow, invasive, and expensive, and are associated with several technical problems including the need to perform the procedures repeatedly because of their quantitative inefficiency. The complication of complement activation on the matrices exists. Overall, therapeutic successes have been modest, at test.

[0005] Another general, non-specific approach involves aggressive immunosuppressive therapy with corticosteroids, and cytotoxic and nonsteroidal antiinflammatory drugs. Although in many instances clinical improvements have been obtained, there continues to be significant morbidity and mortality in autoimmune diseases despite these medications.

[0006] Therefore, notwithstanding these advances in autoimmune therapy, there remains to be seen an internal therapy which is specific for the target pathogenic autoantibody and which is both fast and quantitative.

Disclosure of the Invention

[0007] Accordingly, it is an objective of the present invention to provide an autoimmune therapy which is highly specific for a target pathogenic autoantibody and which produces fast and quantitative results.

[0008] It is a further objective of the present invention to provide such an autoimnune therapy for the treatment of autoimmune diseases in humans and non-human primates.

[0009] The above objectives have been met by the present invention, which are called antigen-based heteropolymers. These are specific for both complement receptor site (CR1) on a primate erythrocyte and a target pathogenic autoantibody. More specifically, the above objectives have been met by the present invention which provides a complex which comprises at least one antigen-based heteropolymer comprising a monoclonal antibody specific for binding to a CR1 site on a primate erythrocyte, wherein the monoclonal antibody is crosslinked to an antigen specific for a target pathogenic autoantibody.

[0010] In addition, the objectives have been met by the present invention which further provides the use of a composition comprising an effective amount of an antigen-based heteropolymer comprised of a monoclonal antibody specific for the CR1 site on a primate erythrocyte, wherein the monoclonal antibody is crosslinked to an antigen which is specific for a target pathogenic autoantibody for the preparation of a medicament for the treatment of an autoimmune disease in a human or non-human primate.

Brief Description of the Invention

[0011] Figure 1 is a schematic illustration of the antigen-based heteropolymer complex consisting of a monoclonal antibody bound to the CR1 receptor site on a primate erythrocyte. The monoclonal antibody is also crosslinked to an autoantigen (dsDNA) which in turn, is bound to the target pathogenic autoantibody (anti-dsDNA antibody).

[0012] Figure 2 is a graph showing the kinetics of the antigen-based heteropolymer-mediated binding of IgG anti-dsDNA antibodies to human red blood cells. The counts bound are proportional to the amount of human IgG bound

(see below). Ma, Mo, and Ha are the last name initials of SLE patients from whom the plasmas or IgG fractions were taken. The solid symbols indicate incubation with red blood cells and antigen-based heteropolymer, and the above respective antibodies. The open symbols represent incubation with red blood cells and antibodies, without antigen-based heteropolymer.

[0013] Figure 3 is a graph showing examination of red blood cells prepared and incubated as described in Figure 2, and probed with ^{125}I-labeled monoclonal antibodies to both human IgG and human IgM. Plasma Ma is representative of samples from patients with severe nephritis and contains almost exclusively IgG anti-dsDNA antibodies at a very high titer. Plasma Va contains both IgG and IgM anti-dsDNA antibodies.

[0014] Figure 4 is a graph showing results of control experiments to demonstrate the specificity of binding. Human red blood cells were examined with a four-fold dilution of plasma Ma, or an IgG fraction from plasma Ma was examined. Pl = plasma; Nl = normal; SRBCS = sheep red blood cells; AHP = antigen-based heteropolymer; Ma = patient with severe nephritis.

[0015] Figure 5 is a graph showing the results of control experiments which indicate that binding is permissive in normal human serum and verify that the red blood cells are not lysed. A two-fold dilution of plasma Ma (diluted in bovine serum albumin) was compared to a similar dilution of plasma Ma in fresh, normal human serum. PL = plasma; NHSC = normal human serum complement; AHP = antigen-based heteropolymer; IRR irrelevant monoclonal antibody.

[0016] Figure 6 is a graph showing the results of dose response experiments illustrating the optimum input of antigen-based heteropolymer to maximize binding of human IgG anti-dsDNA antibodies to 150 µl of red blood cells (50% hematocrit) in an SLE IgG sample. AHP3 was prepared as in Example 1 (see below). AHP2 = contains 1/2 as much dsDNA as AHP3; AHP1 = contains 1/4 as much dsDNA as AHP3.

[0017] Figure 7 is a graph showing binding as a function of the concentration of IgG anti-dsDNA antibodies. A 50% dispersion of human red blood cells was examined in a mixture of bovine serum albumin and IgG Mo.

[0018] The data (calculated line) was fit to a simple straight line equation by the least squares analysis.

[0019] Figure 8 is a graph showing binding as a function of the concentration of IgG anti-dsDNA antibodies using a very high titer anti-dsDNA plasma (Ma).

[0020] Figure 9 is a graph showing binding as a function of the concentration of IgG anti-dsDNA antibodies using plasma Ma. (7G9 AHP and 1B4 kHP alone as compared to cocktail containing 7G9 and 1B4 together.)

Best Mode For Carrying Out The Invention

[0021] The present invention is based on the unique structural and biophysical properties of the primate red blood cell complement receptor (CR1). The antigen-based heteropolymer complexes of the present invention are specific for both the CR1 site on a primate red blood cell and for a target pathogenic autoantibody.

[0022] The antigen-based heteropolymers of the present invention are prepared from monoclonal antibodies which are specific for the CR1 receptor site on a primate erythrocyte. The monoclonal antibodies must also be capable of being crosslinked to an autoantigen which is specific for the target pathogenic autoantibody.

[0023] Examples of such monoclonal antibodies used in the present invention include 1B4, HB8592, and 7G9. HB8592 and 1B4 are disclosed in Taylor et al., "Use of heteropolymeric monoclonal antibodies to attach antigens to the C3b receptor of human erythrocytes: A potential therapeutic treatment," Proc. Nat'l. Acad. Sci., 88:3305-3309 (April 1991); Reist et al., "Antigens pre-bound to the primate erythrocyte complement receptor via crosslinked bispecific monoclonal antibody heteropolymers are rapidly cleared from the circulation, "Eur. J. Immunol. 23:3021-3027 (1993). 7G9 is a mAb recently developed in the inventors' laboratory and can be prepared by known techniques. Other mAbs to CR1 available and useful include 3D9 and E-11 [(previously used by the present inventors in Proc. Nat'l. Acad. Sci., Vol. 91 (July 1992)], and 57F and YZ1 (prepared and reported by Nussenzweig and Fearon, respectively). It is likely that any mAb for CR1 will work successfully in the antigen-based heteropolymer system of the present invention.

[0024] The monoclonal antibody is crosslinked to an antigen specific for a target pathogenic autoantibody. The crosslinking can be performed by any efficacious crosslinking method. For example, purified monoclonal antibodies may first be biotinylated. Typically, each monoclonal antibody may contain 5 biotins. The biotinylated purified monoclonal antibody is then crosslinked to a previously biotinylated antigen or autoantigen by the use of streptavidin. Other known methods, such as use of N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) can be used for crosslinking the monoclonal antibody to the antigen if the antigen has free amino groups. The details of preparation of non-antigen-based heteropolymers can be found in Taylor et al. Proc. Nat'l. Acad. Sci. *supra*; Reist et al. supra; and Taylor et al., "In vitro binding and clearance of circulating antigen by bispecific heteropolymer-mediated binding to primate erythrocyte complement receptor," J. Immunol., 148(8):2462-2468 (April 1992).

[0025] Many different autoantibodies can theoretically be cleared from the circulation of a primate by using the antigen-based heteropolymers of the present invention. Specifically, certain humans with Hemophilia have been shown to be deficient in factor VIII. Recombinant factor VIII replacement treats this hemophilia. However, eventually some patients develop antibodies against factor VIII, thus interfering with the therapy. The antigen-based heteropolymer of

the present invention prepared with factor VIII provides a therapeutic solution for this problem.

[0026] Specifically, an antigen-based heteropolymer comprised of factor VIII crosslinked to a mAb to CR1 specifically binds circulating anti-factor VIII autoantibodies to erythrocyte CR1 and facilitates clearance of said autoantibodies. Factor VIII is allowed to circulate and facilitate blood clotting.

[0027] Other autoantibodies which can be cleared by the antigen-based heteropolymer complex of the present invention include, but are not limited to, autoantibodies to the following antigens: the muscle acetylcholine receptor (the antibodies are associated with the disease myasthenia gravis); cardiolipin (associated with the disease lupus); platelet associated proteins (associated with the disease idiopathic thrombocytopenic purpura); the multiple antigens associated with Sjogren's Syndrome; the antigens implicated in the case of tissue transplantation autoimmune reactions; the antigens found on heart muscle (associated with the disease autoimmune myocarditis); the antigens associated with immune complex mediated kidney disease; the dsDNA and ssDNA antigens (associated with lupus nephritis); desmogleins and desmoplakins (associated with pemphigus and pemphigoid); or any other antigen which is well-characterized, can be crosslinked to an anti-CR1 mAb, and is associated with disease pathogenesis.

[0028] When the above antigen-based heteropolymer complexes are injected into the circulation of a human or non-human primate, the antigen-based heteropolymer will readily bind to the red blood cells via the monoclonal antibody at a high percentage and in agreement with the number of CR1 sites on the red blood cells. At approximately the same rate, the antigen-based heteropolymer will then bind to the autoantibody via the antigen, which is crosslinked to the monoclonal antibody. The red blood cells which contain the bound antigen-based heteropolymer autoantibody complex can then act therapeutically by facilitating the neutralization and clearance from the circulation of the bound pathogenic autoantibody. In the present invention, the antigen-based heteropolymers facilitate binding to the red blood cells and subsequently clear the autoantibody from the circulation of human and non-human primates, without also clearing the red blood cells themselves.

[0029] The present inventors have found for the first time, that by crosslinking anti-CR1 monoclonal antibodies with an autoantigen, thus generating the antigen-based heteropolymers of the present invention, it is possible to bind specifically to red blood cells the vast majority (ca. 80%-95%, Table 1 and Figure 9) of the autoantibodies (specific for an antigen) found in plasmas from patients with a specific autoimmune disease. Specifically, the examples demonstrate that it is possible to bind specifically to red blood cells the vast majority of the autoantibodies to the dsDNA antigen found in plasmas from patients with systemic lupus erythematosus (SLE).

[0030] The present invention utilizes the unique properties of the primate red cell CR1 which enable it to bind and clear complement-opsonized immune complexes from the circulation. The immune complexes cleared via this system are taken up by the liver and spleen (Cornacoff, J. Clin. Invest. (1983)).

[0031] The specificity of the binding of the AHP complex to the autoantibodies or antibodies and the red blood cell CR1-antigen-based heteropolymer complex is clear, because when a) the antigen-based heteropolymer is omitted; b) sheep red blood cells which lack the CR1 site are substituted for human red blood cells; c) the biotinylated autoantigen is omitted in preparing the antigen-based heteropolymer; and d) normal sera or normal IgG is substituted for SLE plasmas, no binding is observed (Figure 4). Further proof of specificity is demonstrated in that no binding is observed if e) the antigen-based heteropolymer is prepared with no monoclonal antibody; f) the antigen-based heteropolymer is prepared with an "irrelevant" monoclonal antibody; and g) excess monomeric monoclonal antibody anti-CR1 is used to inhibit the antigen-based heteropolymer complex (Figure 5). It has also been confirmed that the presence of fresh serum (as a complement source, 25% by volume) does not inhibit specific binding or lyse the red blood cells (Figure 5).

[0032] The input range of the antigen-based heteropolymer is determined based on the red blood cell concentration and the number of CR1 epitope sites recognized by the anti-CR1 monoclonal antibodies per red blood cell. If the antigen-based heteropolymer complex is added in excess, a fraction of the antigen-based heteropolymer will not bind to red blood cells, but will instead inhibit the uptake of the autoantibodies by the red blood cell. The reason is that when the free antigen-based heteropolymer is in solution, it will simply compete for available autoantibody with antigen-based heteropolymer bound to red blood cells. Thus, the antigen-based heteropolymer-mediated binding of the autoantibodies to human red blood cells follows a bell-shaped curve when binding is examined as a function of the input antigen-based heteropolymer concentration (Figure 6).

[0033] The quantitative effectiveness of the antigen-based heteropolymers of the present invention in facilitating the binding of autoantibodies to red blood cells is demonstrated in the results of Farr assays.

[0034] The results of the Farr assays demonstrate that the autoantibodies are indeed specifically absorbed to red blood cells and in a high percentage (> 90% in Table 1) in the presence of the antigen-based heteropolymer complex of the present invention.

[0035] It has further been demonstrated that there is a linear relationship between the input of autoantibodies and the level of autoantibodies present on the red blood cells due to antigen-based heteropolymer-mediated binding (Figure 7). In some instances the system shows a saturation because the concentration of the autoantibodies in the plasma is so high that even at the optimum input of antigen-based heteropolymers, not all of the autoantibodies can be bound to the red blood cells under standard conditions (Figure 8). This is also shown in the Farr assays. For example, for a

very high titer sera, a fraction of the autoantibody binding activity is not bound to the red blood cells (Table 1) due to its high concentration. These cases are the exception, and for the majority of samples from patients with lupus nephritis (which have pathogenic levels of anti-dsDNA antibodies), the method of the present invention removes > 90% of the anti-dsDNA antibodies.

[0036] However, the saturation problem can be solved by using combinations of antigen-based heteropolymer which contain monoclonal antibodies that bind to different sites on CR1. The monoclonal antibodies 7G9 and 1B4 bind to separate and noncompeting sites on red blood cell CR1. Therefore, a "cocktail" containing a mixture of two antigen-based heteropolymers made with respective monoclonal antibodies gives rise to greater binding of autoantibodies to red blood cells. Plasma Ma is one of the highest titer SLE IgG anti-dsDNA plasma studied. The single antigen-based heteropolymer prepared with either anti-CR1 monoclonal antibodies 7G9 or 1B4 alone cannot absorb all anti-dsDNA antibodies to red blood cells in the undiluted plasma. However, when the "cocktail" is used, more than 90% of the IgG anti-dsDNA in plasma Ma is absorbed to red blood cells (Figure 9). Also, the results of the Farr assay (Table 1) of the plasma supernatants indicate that greater than 90% of the anti-dsDNA antibody binding activity in plasma Ma is removed when it is treated with the antigen-based heteropolymer cocktail and red blood cells.

[0037] The present invention further encompasses the use of a composition comprising an effective amount of an antigen-based heteropolymer complex of the present invention for the preparation of a medicament for treating an autoimmune disease in a primate. The route of administration will likely be by intravenous injection into the blood of a human or non-human primate.

[0038] An effective amount of the antigen-based heteropolymer complex of the present invention is from 1-10 mg, preferably 5 mg administered once. This dosage should clear up to 2 μg/ml of the autoantibody from the circulation of a primate. In a therapeutic environment, the administration should be repeated until complete clearance of the pathogenic autoantibody. The antigen-based heteropolymers of the present invention can be used in combination with certain fluids used for intravenous infusions.

[0039] Although the prototype studies disclosed herein are carried out using mouse mAbs, currently available technology should allow "humanization" of these mouse mAbs. This will decrease the chance that an immune response to the antigen-based heteropolymer will abrogate its effectiveness in repeated doses.

[0040] Red blood cells removed and isolated may also be used as therapeutic agents. Once franked with the antigen-based heteropolymer, these red blood cells can be reintroduced into the patient, where, in the bloodstream free autoantibodies will bind and be immobilized on the red blood cell, and subsequently cleared in accordance with the body's red blood cell clearing mechanism.

[0041] As set forth above, in an alternative embodiment, red blood cells are franked with a "cocktail" of at least two antigen-based heteropolymers, which, in addition to binding to the target autoantibody, also bind to several distinct and non-overlapping sites on CR1 of the primate red blood cell. As some of the following experiments show, by using at least two non-overlapping monoclonal antibodies for binding to CR1 on the red blood cell of primates, the number of heteropolymer complexes that can also bind is increased at a high percentage and in good agreement with the number of available binding sites. This, in turn, allows for more autoantibody binding to the heteropolymer complexes of the present invention. This augments the capability of a relatively small number of red blood cells to bind to a relatively larger amount of autoantibody, and further can facilitate the removal of the autoantibody through the normal primate immune clearance system.

[0042] The AHP of the present invention can further be used in the case of clearance of exogenous administered antibodies which become pathogenic.

EXAMPLES

EXAMPLE 1

Monoclonal antibodies and crosslinked AHP anti-CR1/dSDNA complexes.

[0043] Three monoclonal antibodies specific for primate CR1, i.e., 1B4, HB8592, and 7G9, were purified by known methods as set forth in Reist et al., *supra*. The purified monoclonal antibodies were then biotinylated by known methods. Typically, each monoclonal antibody would contain 5 biotins. An amount of 24 μg of biotinylated 7G9 was incubated *for* 30 minutes at room temperature with 30 μg of streptavidin (SA) in a volume of 48 μl borate saline (BS buffer).

[0044] The resulting sample was then incubated with 33 μg of biotinylated dsDNA which was purchased from Dr. W. Emlen of the University of Colorado Medical Center (1 biotin per 30 base pairs in 1 ml of BS buffer) in order to crosslink 7G9 to the biotinylated dsDNA. The process was repeated using 1B4 or HB8592. The anti-CR1/dsDNA complexes formed were used without any other purification.

EXAMPLE 2

Binding assays.

[0045] SLE plasmas or IgG fractions from 3 different patients which are denoted by last name initials (i.e., Ma, Mo, and Ha) were incubated by general methods [Taylor et al., "The Interaction of Antibody/DNA Immune Complexes with complement, "Arthritis and Rheumatism, 30(2):176-185 (February 1987) with red blood cells and AHP (10 μl AHP3 per 150 μl of 50% red cells) (solid symbols in Figure 2) and without AHP (open symbols in Figure 2) for the indicated time period at 37°C as follows: Type O red blood cells were washed and then reconstituted to a 50% hematocrit in one of the above plasmas. The AHP was added in an "equivalence" of ca. 0.6-1.2 μg of anti-CR1 mAb per ml blood based on 500-1000 CR1 receptors per red blood cell. After brief incubation, the sample was centrifuged and the supernatant isolated and saved for an independent assay (Farr assay) of the anti-dsDNA antibodies. The red cell pellets were washed 3 times and then an ^{125}I-labeled monoclonal antibody to human IgG (HB43) was added to the red blood cells to measure uptake of human IgG. (Taylor et al., 88:3305-3309, Proc. Natl. Acad. Sci. *supra.*) The arrow at the bottom of Figure 2 emphasizes the low level of background binding to red blood cells of all 3 samples after a full 15 minute incubation. The broken lines at the bottom left of the figure are extrapolations to 0 minutes of incubation with the AHP.

[0046] Figure 2 demonstrates the results of the kinetics of AHP-mediated binding of IgG anti-dsDNA antibodies to human red blood cells. These results show that the complexes of the present invention bind to red blood cells. The results further show that by virtue of the incorporated dsDNA, the AHP complexes facilitate the specific and rapid binding (ca. 80% complete in ca. 5 minutes at 37°C as shown in Figure 2) to red blood cells of high avidity IgG and/or IgM/anti-dsDNA antibodies found in SLE plasmas. The specificity of the binding for anti-dsDNA antibodies and the anti-CR1/dsDNA AHP complex is clear due to the fact that all the binding is abrogated when the AHP is omitted (open symbols in Figure 2).

EXAMPLE 3

[0047] Two SLE samples were prepared as in Example 2 above. However, the samples were incubated with AHP for 15 minutes at 37°C.

[0048] Plasma Ma, as shown in Figure 3, is representative of samples from patients with severe nephritis, and as previously reported, has almost exclusively IgG anti-dsDNA antibodies at very high titer (Taylor et al., Arthritis and Rheumatism, (1987)]. The plasma Va was shown in Taylor et al. to have both IgG and IgM anti-dsDNA antibodies.

[0049] The red blood cells were probed with ^{125}I-labeled monoclonal antibodies to both human IgG (HB43) and human IgM (HB57).

EXAMPLE 4

Control experiments to demonstrate the specificity of binding.

[0050] Plasma Ma, or an IgG fraction from plasma Ma, was prepared as set forth above. The plasma Ma and the IgG fraction from plasma Ma were diluted four-fold. Human red blood cells were used as above in this experiment. Five different samples were prepared as follows: a) AHP omitted; b) sheep red blood cells (SRBCS) lacking the CR1 site were substituted for human red blood cells; c) the biotinylated dsDNA was omitted in preparing the AHP; and d) normal plasma or normal IgG was substituted for SLE plasma (Figure 4).

[0051] The results shown in Figure 4 demonstrate that only in the presence of the AHP and human red blood cells (first 2 groups of Figure 4 on the left) is binding evident. If AHP is omitted or the dsDNA antigen is omitted from the AHP, no binding is seen. Further, when sheep red blood cells were used with Ma, or normal human IgG was used in place of Ma, binding was also reduced to background levels.

EXAMPLE 5

[0052] In this experiment, a two-fold dilution of plasma Ma (diluted in bovine serum albumin) was compared to the same dilution of plasma Ma in fresh normal human serum. The following four samples were prepared: a) the AHP was prepared with no monoclonal antibody; b) the AHP was prepared with a monoclonal antibody to the dinitrophenol group (23D1, an irrelevant (IRR) antibody); c) excess monomeric 7G9 anti-CR1 was used to inhibit the 7G9/dsDNA AHP; and d) serum prepared with AHP.

[0053] The proof of specificity is demonstrated in Figure 5 by the showing that no binding is observed if the AHP is prepared with no monoclonal antibody, the AHP is prepared with an irrelevant monoclonal antibody, or if excess monomeric 7G9 anti-CR1 is used to inhibit the 7G9/dsDNA AHP. The specificity of the AHP, which contains biotinylated

monoclonal antibody 7G9, is demonstrated because excess monoclonal antibody 7G9 is able to compete for sites on CR1 and abrogate all binding. It has also been confirmed that the presence of fresh serum (as a complement source, 25% by volume) does not inhibit specific binding or lyse the red blood cells (Figure 5).

EXAMPLE 6

Farr assay showing quantitative effectiveness of the antigen-based heteropolymers.

[0054] The quantitative effectiveness of the antigen-based heteropolymer complexes of the present invention in facilitating binding of target autoantibodies to red blood cells is demonstrated in Table I which shows the results of Farr assays. The Farr assay was performed as in Taylor et al., Arthritis and Rheumatism, *supra*. These assays are specific for anti-dsDNA antibodies. The supernatant samples contain the plasma proteins and the vast majority of IgG, not specific for dsDNA, which did not bind to red blood cells. These assays indicate that the anti-dsDNA binding activity in the supernatants is reduced considerably for most SLE plasma and IgG fractions (more than 90% in Table 1). That is, the IgG anti-dsDNA antibodies are indeed specifically absorbed to the red blood cells in the presence of the AHP complex.

Table 1

Efficiency of Removal of Anti-dsDNA Antibody Binding Activity from SLE Plasmas by adsorption to Red cells in the Presence of a Specific AHP. Analysis of binding of ^{3}H-dsDNA in the Farr Assay.				
		^{3}H-dsDNA (DPM) in the SN (Unbound Counts)		
SLE Plasma[a]	Dilution[b] tested in Farr assay	Adsorbed with red cells alone	Adsorbed with red cells +AHP	% Adsorbed[c]
Ma/2	5 25 100	58 270 NL IgG[d]: (1000)	710 950 970	95
Va/1	10 25 100	680 1040 1420 NL IgG[d]: (1800)	1400	90
Co/1	4 20 40	730 1280 1550 NL IgG[d]: (2600)	1250 1830 2300	80
Ha/2	5 20 80 320	30 90 270 390 NL IgG[d]: (1200)	120 360 630 680	85, 95
Ma/1	4	370	1760	90[e]

a) Dilution of SLE plasma incubated with the red cells (± AHP).

b) Dilutions of material not bound to red cells tested in Farr assay.

c) Percent adsorbed calculated based on relative dilution of "+AHP" sample needed to give same level of binding as sample lacking AHP. Unless otherwise specified, 7G9 was used to generate the AHP.

d) In Farr assays, IgG antibodies are precipitated with saturate ammonium sulfate, and then dsDNA not bound to the anti-dsDNA antibodies is measured in the supernatant (SN). The background binding level for normal IgG is given for each assay. A number of dsDNA preparations of different input and specific activity were tested.

e) A "cocktail" of the AEP prepared with both 7G9 and 1B4 was tested. When the two AHP's were each evaluated separately, only 70% of the anti-dsDNA binding activity was adsorbed.

Table 1 (continued)

Efficiency of Removal of Anti-dsDNA Antibody Binding Activity from SLE Plasmas by adsorption to Red cells in the Presence of a Specific AHP. Analysis of binding of ^{3}H-dsDNA in the Farr Assay.				
	^{3}H-dsDNA (DPM) in the SN (Unbound Counts)			
SLE Plasma[a]	Dilution[b] tested in Farr assay	Adsorbed with red cells alone	Adsorbed with red cells +AHP	% Adsorbed[c]
	8 16 32	470 970 1700 NL IgG[d]: (3000)		

a) Dilution of SLE plasma incubated with the red cells (± AHP).

b) Dilutions of material not bound to red cells tested in Farr assay.

c) Percent adsorbed calculated based on relative dilution of "+AHP" sample needed to give same level of binding as sample lacking AHP. Unless otherwise specified, 7G9 was used to generate the AHP.

d) In Farr assays, IgG antibodies are precipitated with saturate ammonium sulfate, and then dsDNA not bound to the anti-dsDNA antibodies is measured in the supernatant (SN). The background binding level for normal IgG is given for each assay. A number of dsDNA preparations of different input and specific activity were tested.

[0055] The above results demonstrate that the autoantibodies are indeed specifically absorbed to red blood cells and in a high percentage (> 90%) in the presence of the antigen-based heteropolymer complex of the present invention.

EXAMPLE 7

Dose response experiments.

[0056] AMP1, AHP2 and AHP3 were prepared as follows. AHP3 was prepared in accordance with the procedures set forth in Example 1 using 10 µl of AHP3 per 150 µl of 50% red blood cells. AHP1 and AHP2 were prepared accordingly, except that AHP2 contains 1/2 as much dsDNA as AHP3, and AHP1 contains 1/4 as much dsDNA as AHP3.

[0057] Dose response experiments were performed to find the optimum input of AHP to maximize binding of human IgG anti-dsDNA antibodies to 150 µl of human red blood cells (50% hematocrit) in an SLE IgG sample.

[0058] The AHP-mediated binding of IgG anti-dsDNA antibodies to human red blood cells follows a bell-shaped curve when binding is examined as a function of the input AHP concentration (Figure 6).

[0059] AHP3 was chosen as the exemplified AHP because it facilitates the highest level of binding IgG anti-dsDNA to red blood cells.

[0060] This data shows that the input range of AHP is determined based on the red blood cell concentration and the number of CR1 epitope sites (recognized by the anti-CR1 monoclonal antibodies) per red blood cell. It is clear that if the AHP is added in excess, a fraction of the AHP will not bind to the red blood cells and will instead, inhibit uptake of the autoantibodies by the red blood cell. The reason is that the free AHP in solution will simply compete for available autoantibody with AHP bound to red blood cells.

EXAMPLE 8

Dose response experiment as a function of autoantibody concentration.

[0061] A 50% dispersion of human red blood cells was examined in mixtures of bovine serum albumin and IgG Mo. Figure 7 shows the results of this binding experiment. The results indicate that there is a linear relationship between counts bound (level of bound IgG) and the input level of SLE IgG anti-dsDNA antibodies due to AHP-mediated binding. The data was calculated to fit a simple straight line equation by least squares analysis.

EXAMPLE 9

[0062] A similar experiment as in experiment 8 was performed except, in this case, a very high titer anti-dsDNA plasma (Ma) was examined. When the sample was used and diluted, it was clear that not all the endogenous IgG anti-dsDNA is bound by the red blood cells. Therefore, in some instances, the system shows saturation because the concentration of IgG anti-dsDNA antibodies in the SLE plasma is so high that even at optimum input of AHP, not all the

IgG anti-dsDNA antibodies can be bound to the red blood cells under standard conditions (Figure 8). This is also shown in the Farr assays (below) where it has been found that for very high titer plasma, a substantial fraction of the anti-dsDNA antibody binding activity is not bound to the red blood cells (see Table 1). Fortunately, these cases are the exceptions. For the majority of SLE plasma from patients with lupus nephritis (and pathogenic levels of anti-dsDNA antibodies) which were examined, the method does remove greater than 90% of the anti-dsDNA antibodies.

EXAMPLE 10

Binding as a function of the concentration of IgG anti-dsDNA antibodies using ARP "cocktail."

[0063] Combinations of AHP were prepared which contain monoclonal antibodies that bind to different sites on CR1. The monoclonal antibodies 7G9 and 1B4 bind to separate and non-competing sites on the CR1 site of the red blood cell. These cocktails produced contain a mixture of the two AHP's made with these respective monoclonal antibodies. The results as shown in Figure 9 show that use of a "cocktail" gives rise to even greater binding of IgG anti-dsDNA antibodies to red blood cells.

[0064] As set forth above, plasma Ma is one of the highest titer SLE IgG anti-dsDNA plasma which has been studied. The single AHP prepared with either anti-CR1 monoclonal antibody 7G9 or 1B4 alone cannot absorb all the anti-dsDNA antibodies to red blood cells in the undiluted plasma. However, when the "cocktail" is used, the continued linearity in the binding (even in the undiluted plasma) suggests that it is possible to absorb to red blood cells, more than 90% of the IgG anti-dsDNA in plasma Ma (Figure 9). This was confirmed in the Farr assays (see Table 1) of the plasma supernatants which indicated that greater than 90% of the anti-dsDNA antibody binding activity in plasma ma is removed when it is treated with the AHP "cocktail" and human red blood cells.

[0065] The AHP prepared with anti-CR1 monoclonal antibody 1B4 showed slightly better binding than the AHP prepared with monoclonal antibody 7G9. However, saturation at the highest input of Ma was seen in both cases. When the cocktail containing both AHP's was used, on the other hand, binding was enhanced considerably which suggests that all the IgG anti-dsDNA can be bound to red blood cells (Figure 9).

Claims

1. An antigen-based heteropolymer (AHP) complex comprising a monoclonal antibody specific for binding to complement receptor (CR1) site on a primate erythrocyte, wherein said monoclonal antibody is crosslinked to an antigen specific for a target pathogenic antibody or autoantibody.

2. The AHP of claim 1, wherein the monoclonal antibody is produced by the hybridoma deposited with the ATCC and assigned accession number HB8592.

3. The AHP of claim 1, wherein the target antibody or autoantibody is selected from the group consisting of antibodies or autoantibodies to the following antigens : factor VIII, muscle acetylcholine receptor, cardiolipin, platelet associated proteins, antigens associated with Sjogren's Syndrome, double stranded deoxyribonucleic acid (dsDNA), and single stranded DNA (ssDNA).

4. The AHP of claim 1, wherein said antigen is selected from the group consisting of factor VIII, muscle acetylcholine receptor, cardiolipin, platelet associated proteins, antigens associated with Sjogren's Syndrome, double stranded deoxyribonucleic acid (dsDNA), and single stranded DNA (ssDNA).

5. The AHP of any one of claims 1-4 wherein the AHP is franked with a human or non-human primate erythrocyte.

6. An AHP cocktail, comprising at least two AHPs according to anyone of claims 1 to 5, wherein each AHP comprises a monoclonal antibody specific for binding to at least two distinct and non-overlapping sites on complement receptor (CR1) on a primate erythrocyte, wherein said monoclonal antibody is crosslinked to an antigen specific for a target pathogenic antibody or autoantibody.

7. Pharmaceutical composition **characterized in that** it comprises an AHP or an AHP cocktail according to any one of claims I to 6.

8. Use of a composition comprising the AHP or the AHP cocktail according to any of claims 1 to 6 for the manufacture of a medicament for the treatment of an autoimmune disease.

Patentansprüche

1. Auf einem Antigen-basierender Heteropolymer(AHP)-Komplex, der einen monoklonalen Antikörper, der für eine Bindung an eine Complement-Rezeptor(CR1)-Stelle an einem Primaten-Erythrocyten spezifisch ist, umfasst, wobei der monoklonale Antikörper mit einem Antigen, das für einen pathogenen Ziel-Antikörper oder -Autoantikörper spezifisch ist, vernetzt ist.

2. AHP nach Anspruch 1, wobei der monoklonale Antikörper durch das Hybridom produziert wird, das bei der ATCC hinterlegt ist und dem die Hinterlegungsnummer HB8592 zugeordnet ist.

3. AHP nach Anspruch 1, wobei der Ziel-Antikörper oder -Autoantikörper aus der Gruppe, bestehend aus Antikörpern oder Autoantikörpern auf die folgenden Antigene, ausgewählt ist: Faktor VIII, Muskel-Acetylcholinrezeptor, Cardiolipin, Blutplättchen-assoziierte Proteine, Antigene, die mit Sjogren-Syndrom assoziiert sind, doppelsträngige Desoxyribonukleinsäure (dsDNA) und einzelsträngige DNA (ssDNA).

4. AHP nach Anspruch 1, wobei das Antigen aus der Gruppe, bestehend aus Faktor VIII, Muskel-Acetylcholin-Rezeptor, Cardiolipin, Blutplättchen-assoziierten Proteinen, Antigenen, die mit Sjogren-Syndrom assoziiert sind, doppelsträngiger Desoxyribonukleinsäure (dsDNA) und einzelsträngiger DNA (ssDNA), ausgewählt ist.

5. AHP nach einem der Ansprüche 1 bis 4, wobei der AHP an einen humanen oder nichthumanen Primaten-Erythrocyten gebunden (franked) ist.

6. AHP-Cocktail, umfassend mindestens zwei AHPs nach einem der Ansprüche 1 bis 5, wobei jeder AHP einen monoklonalen Antikörper, der für eine Bindung an mindestens zwei getrennte und nichtüberlappende Stellen an Complement-Rezeptor (CR1) an einem Primaten-Erythrocyten spezifisch ist, umfasst, wobei der monoklonale Antikörper mit einem Antigen, das für einen pathogenen Ziel-Antikörper oder -Autoantikörper spezifisch ist, vernetzt ist.

7. Pharmazeutische Zusammensetzung, **dadurch gekennzeichnet, dass** sie einen AHP oder einen AHP-Cocktail nach einem der Ansprüche 1 bis 6 umfasst.

8. Verwendung einer Zusammensetzung, die den AHP oder den AHP-Cocktail nach einem der Ansprüche 1 bis 6 umfasst, zur Herstellung eines Medikaments für die Behandlung einer Autoimmunkrankheit.

Revendications

1. Complexe d'hétéropolymères à base d' antigène (HPA) comprenant un anticorps monoclonal se fixant spécifiquement à un site du récepteur du complément (CR1) d'un érythrocyte de primate, dans lequel ledit anticorps monoclonal établit une liaison transversale avec un antigène spécifique d'un anticorps ou d'un auto-anticorps pathogène cible.

2. HPA selon la revendication 1, dans lequel l'anticorps monoclonal est produit par l'hybridome déposé à l'ATCC et dont le numéro de référence attribué est HB8592.

3. HPA selon la revendication 1, dans lequel l'anticorps ou auto-anticorps cible est choisi dans le groupe consistant en les anticorps et auto-anticorps dirigés contre les antigènes suivants : facteur VIII, récepteur musculaire de l'acétylcholine, cardiolipide, protéines associées aux plaquettes, antigènes associés au syndrome de Goujerot-Sjögren, acide désoxyribonucléique double brin (ADNdb), et acide désoxyribonucléique simple brin (ADNsb).

4. HPA selon la revendication 1, dans lequel ledit antigène est choisi dans le groupe consistant en le facteur VIII, le récepteur musculaire de l'acétylcholine, le cardiolipide, les protéines associées aux plaquettes, les antigènes associés au syndrome de Goujerot-Sjögren, l'acide désoxyribonucléique double brin (ADNdb), et l'acide désoxyribonucléique simple brin (ADNsb).

5. HPA selon l'une quelconque des revendications 1 à 4, dans lequel l'HPA est assigné à un érythrocyte de primate humain ou non-humain.

6. Mélange d'HPA, comprenant au moins 2 HPA selon l'une quelconque des revendications 1 à 5, dans lequel chaque HPA comprend un anticorps monoclonal se fixant spécifiquement à au moins deux sites distincts et qui ne se chevauchent pas sur le récepteur du complément (CR1) sur un érythrocyte de primate, dans lequel ledit anticorps monoclonal établit une liaison transversale avec un antigène spécifique d'un anticorps ou d'un auto-anticorps pathogène cible.

7. Composition pharmaceutique **caractérisée en ce qu**'elle comprend un HPA ou un mélange d'HPA selon l'une quelconque des revendications 1 à 6.

8. Utilisation d'une composition comprenant l'HPA ou le mélange d'HPA selon l'une quelconque des revendications 1 à 6 pour la fabrication d'un médicament pour le traitement d'une maladie autoimmune.

FIG. 1

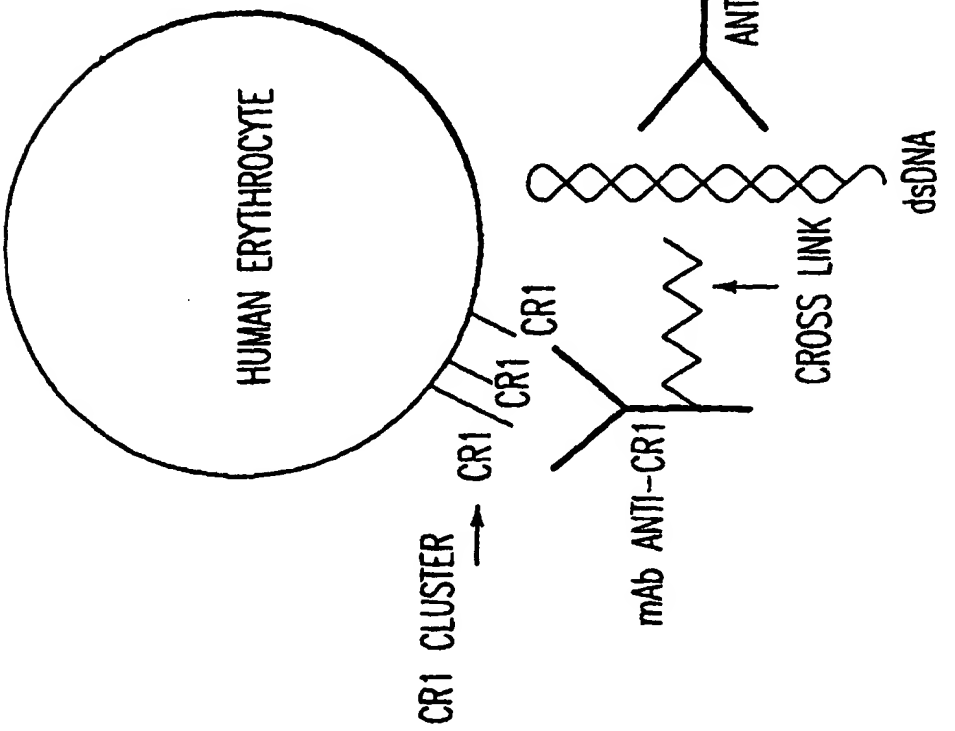
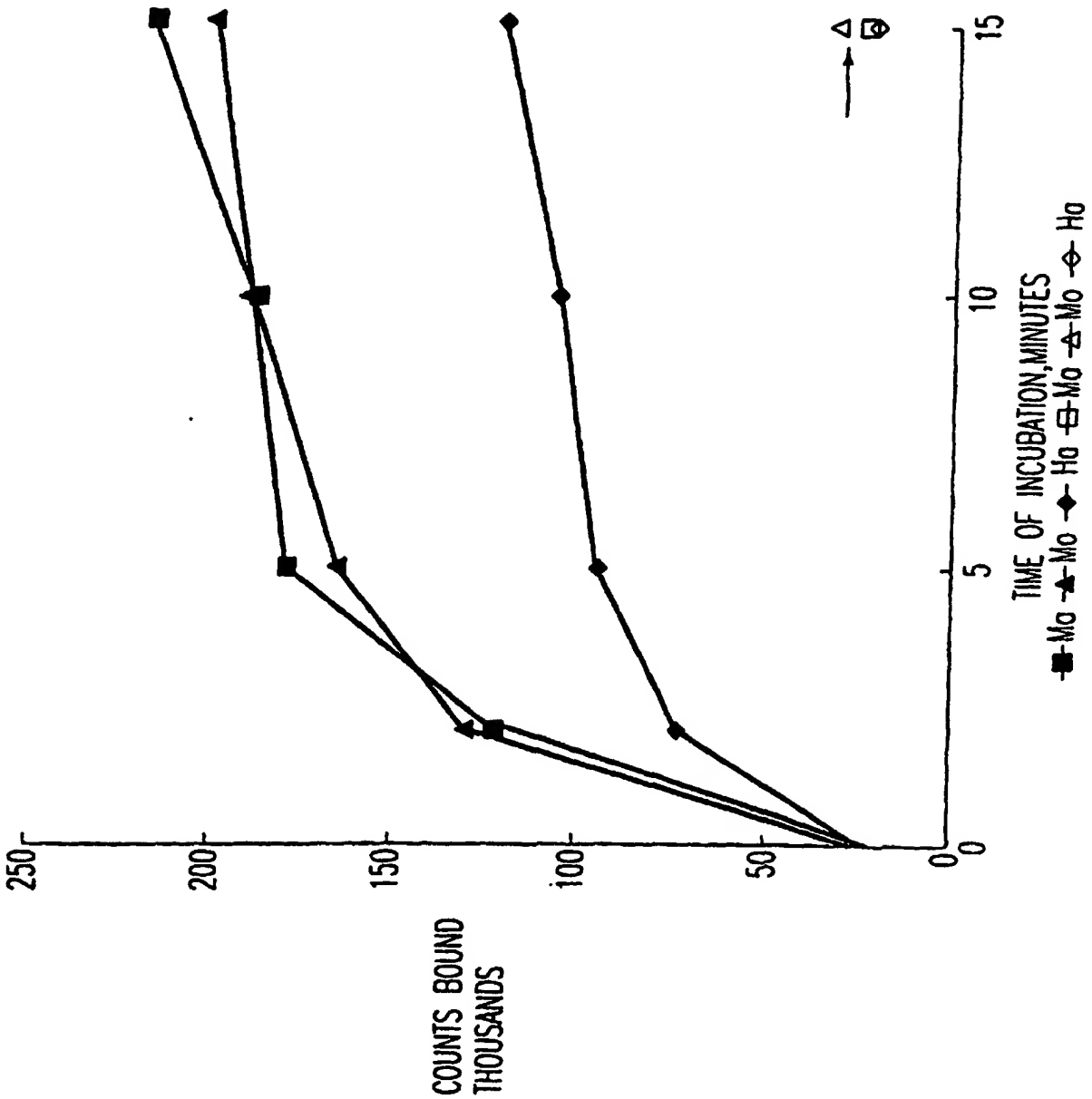

HUMAN ERYTHROCYTE
CR1
CR1
CR1
CR1 CLUSTER
mAb ANTI-CR1
CROSS LINK
dsDNA
ANTI-dsDNA Ab

FIG. 2


250
200
150
100
50
0
0
5
10
15
COUNTS BOUND
THOUSANDS
TIME OF INCUBATION, MINUTES
Mo
Mo
Ho
Mo
Mo
Ho

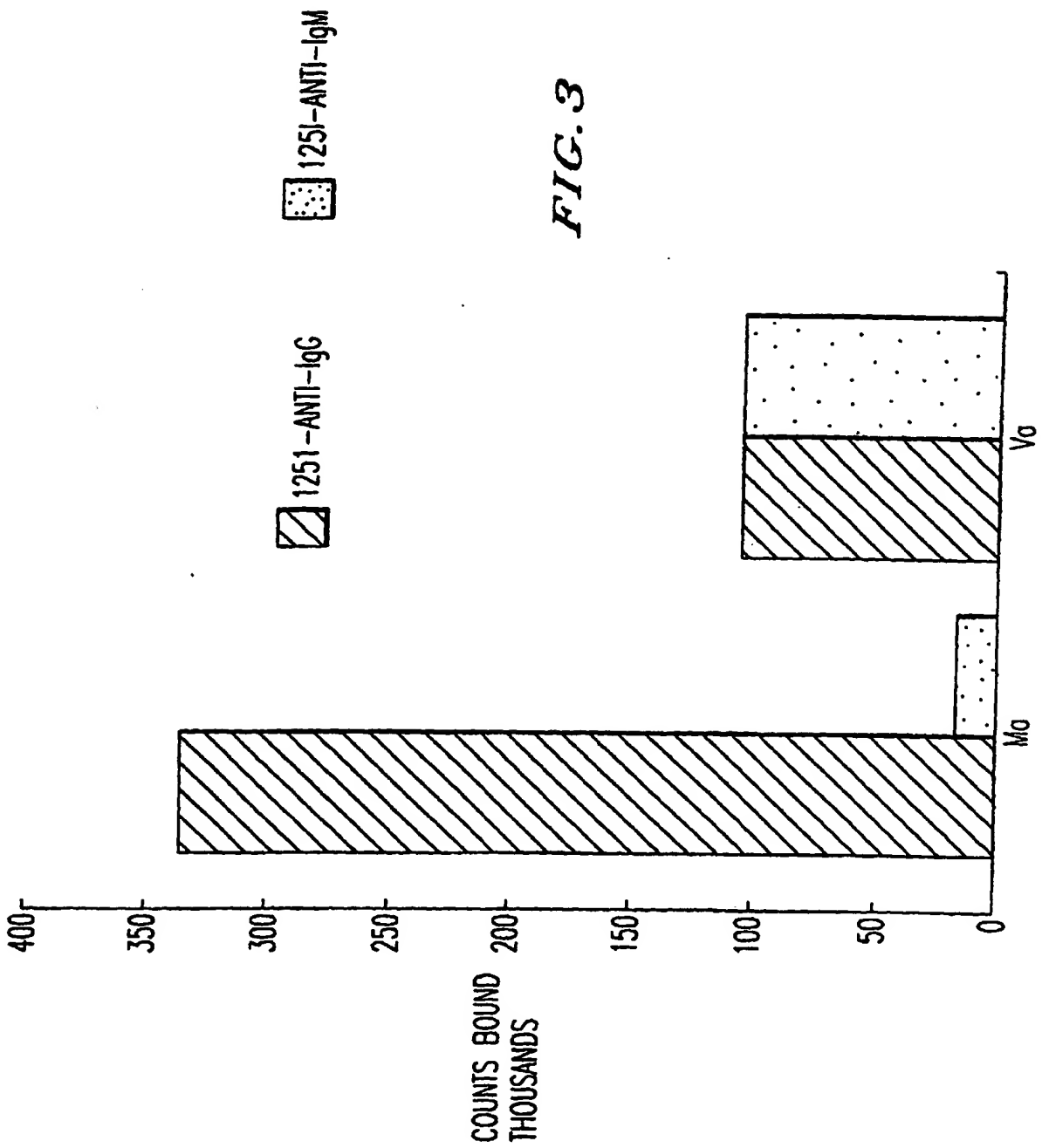
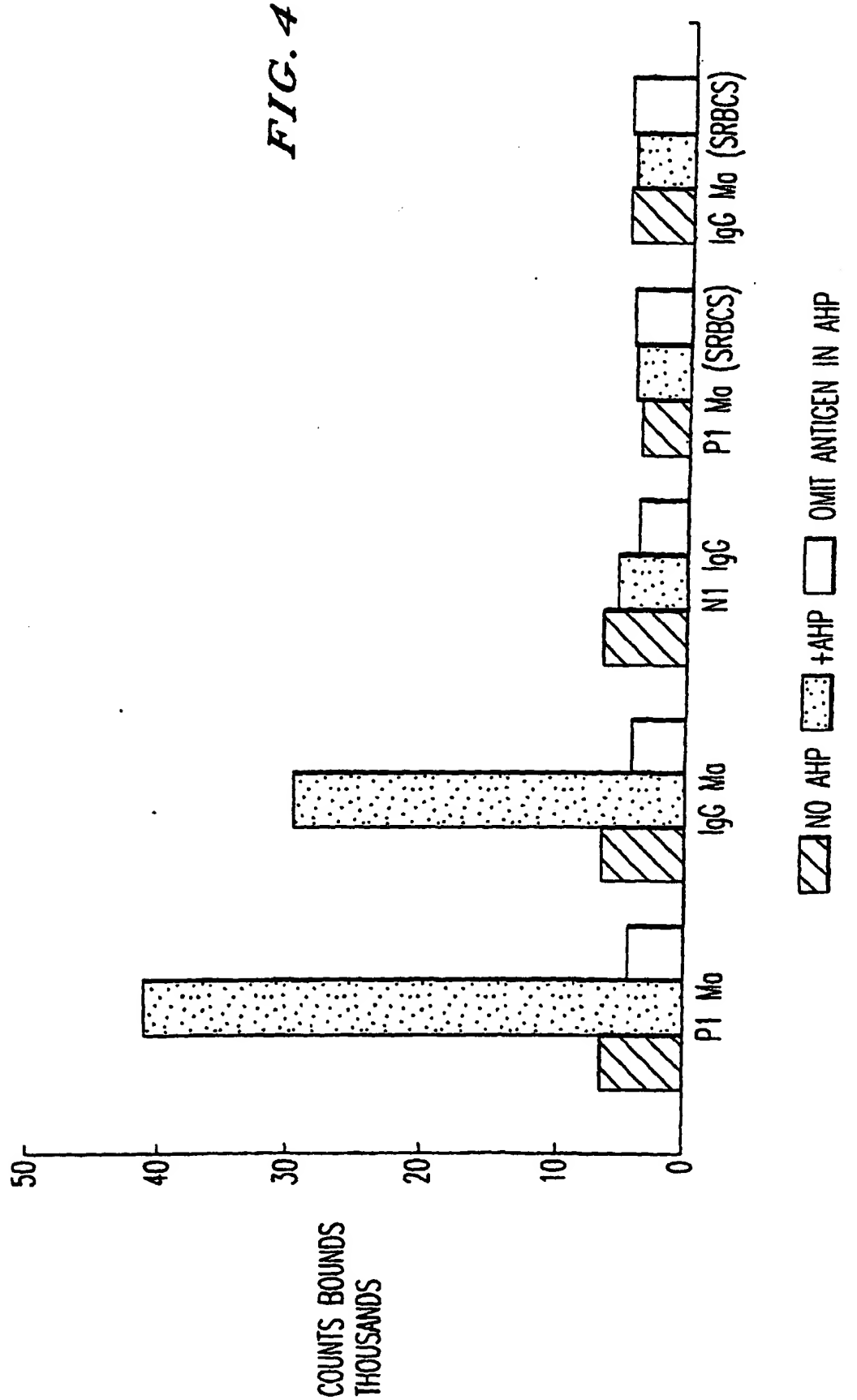

125I-ANTI-IgM
125I-ANTI-IgG
COUNTS BOUND
THOUSANDS
400
350
300
250
200
150
100
50
0
Mo
Vo

FIG. 3

FIG. 4


COUNTS BOUNDS
THOUSANDS
50
40
30
20
10
0
P1 Mo
IgG Mo
N1 IgG
P1 Mo (SRBCS)
IgG Mo (SRBCS)
NO AHP
+AHP
OMIT ANTIGEN IN AHP

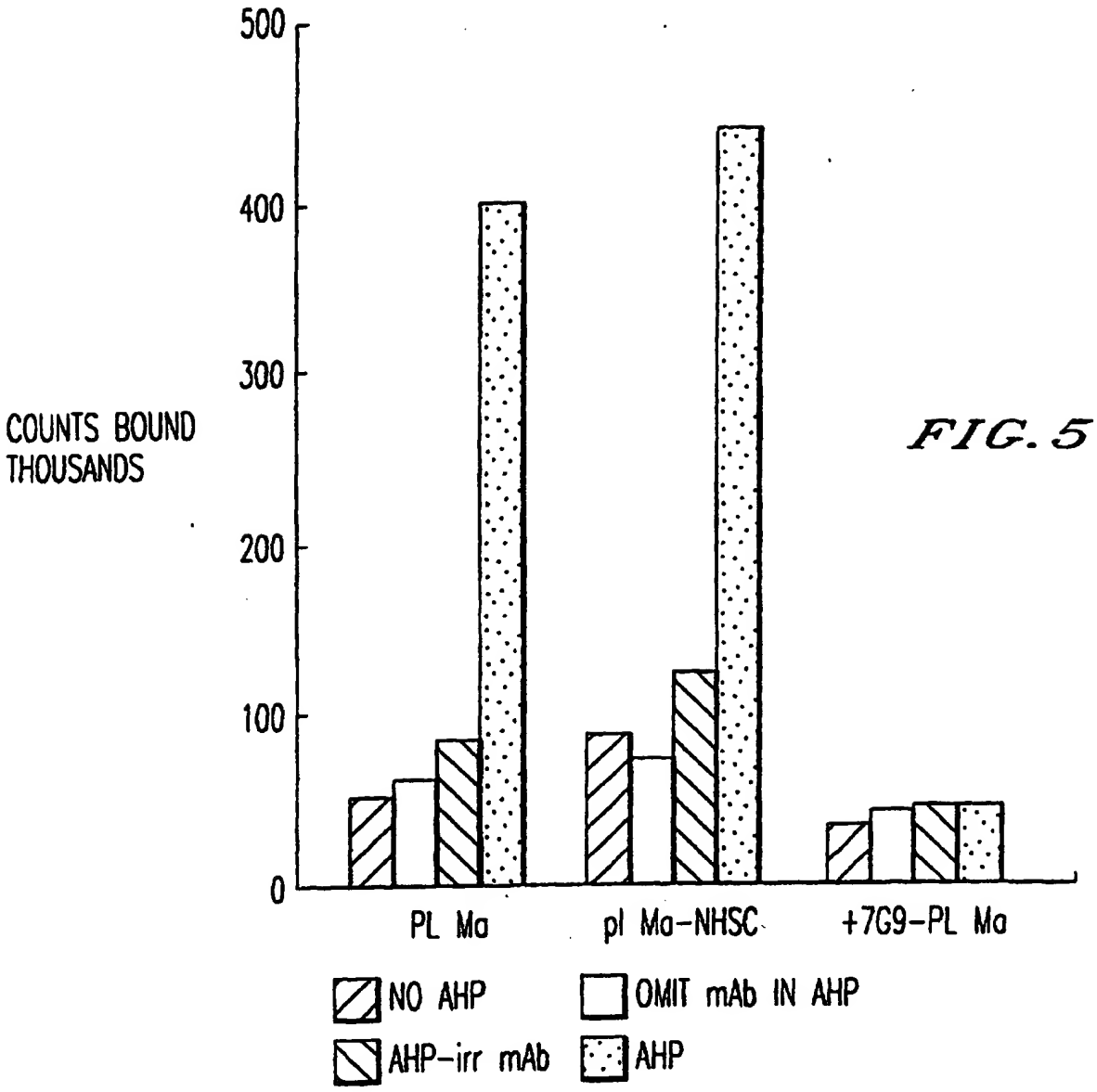

COUNTS BOUND
THOUSANDS
500
400
300
200
100
0
PL Mo
pl Mo-NHSC
+7G9-PL Mo
NO AHP
OMIT mAb IN AHP
AHP-irr mAb
AHP

FIG. 5

FIG. 6

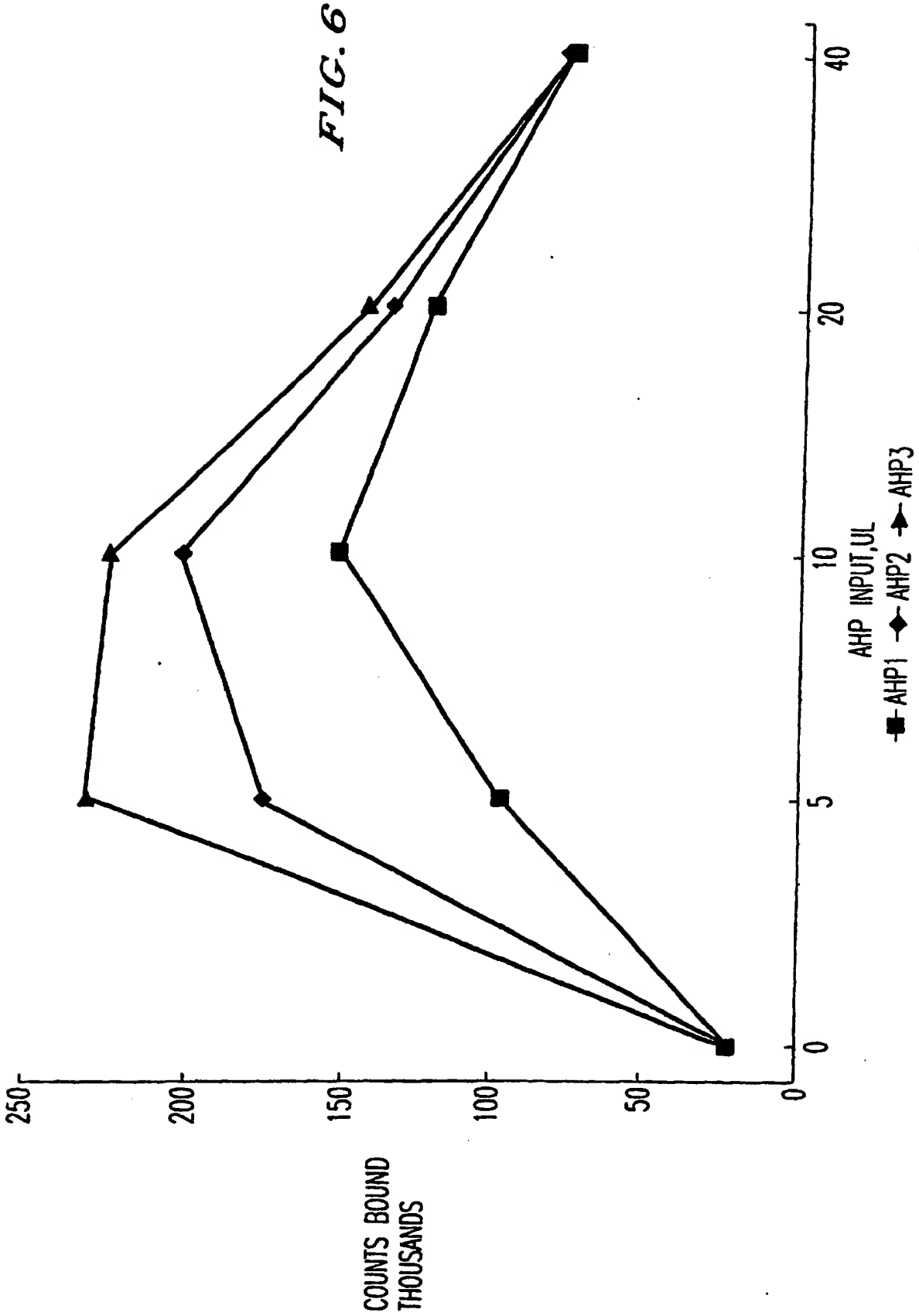
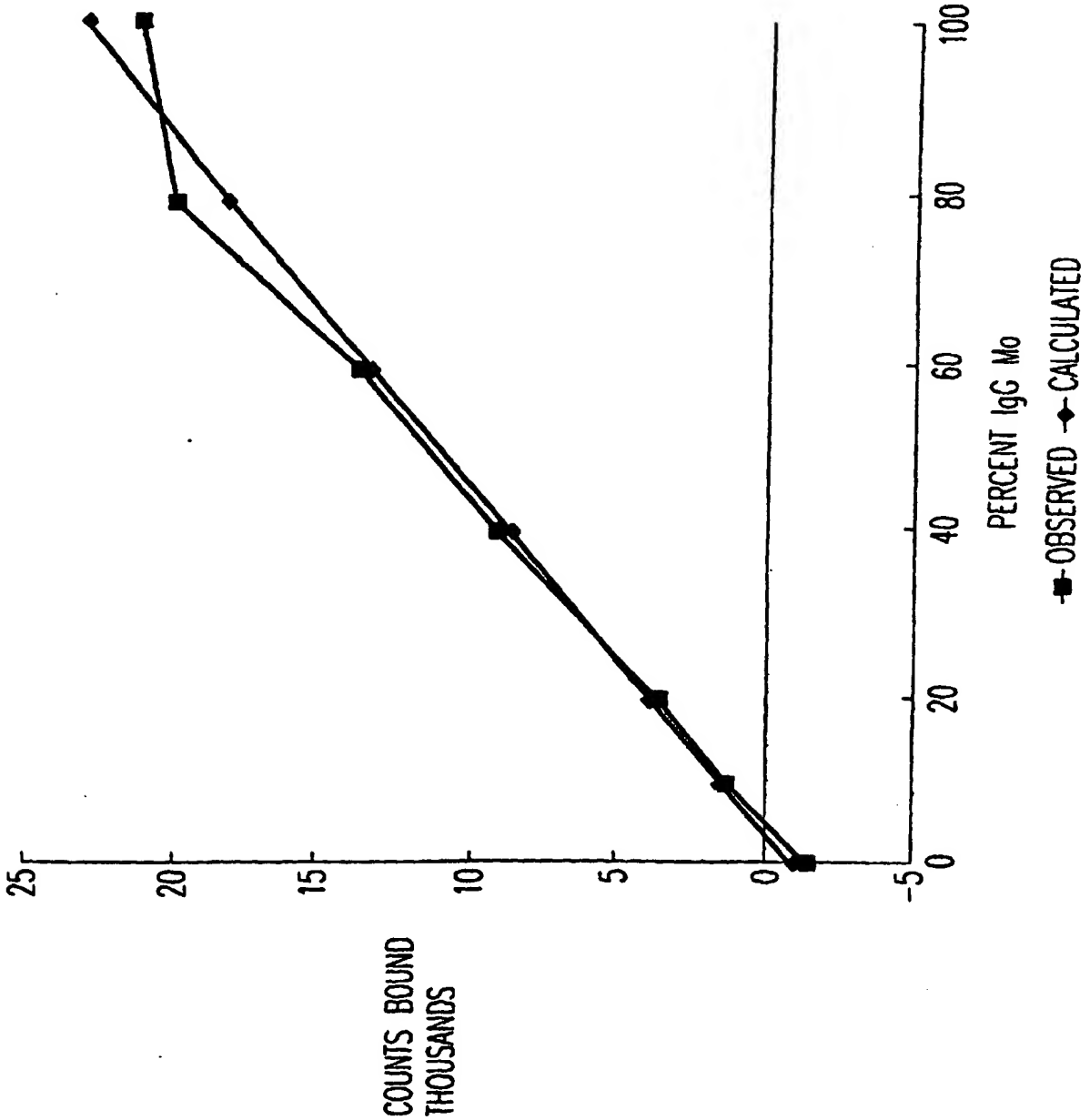

250
200
150
100
50
0
COUNTS BOUND
THOUSANDS
0
5
10
20
40
AHP INPUT,UL
AHP1
AHP2
AHP3

FIG. 7


COUNTS BOUND
THOUSANDS
25
20
15
10
5
0
-5
0
20
40
60
80
100
PERCENT IgG Mo
OBSERVED
CALCULATED

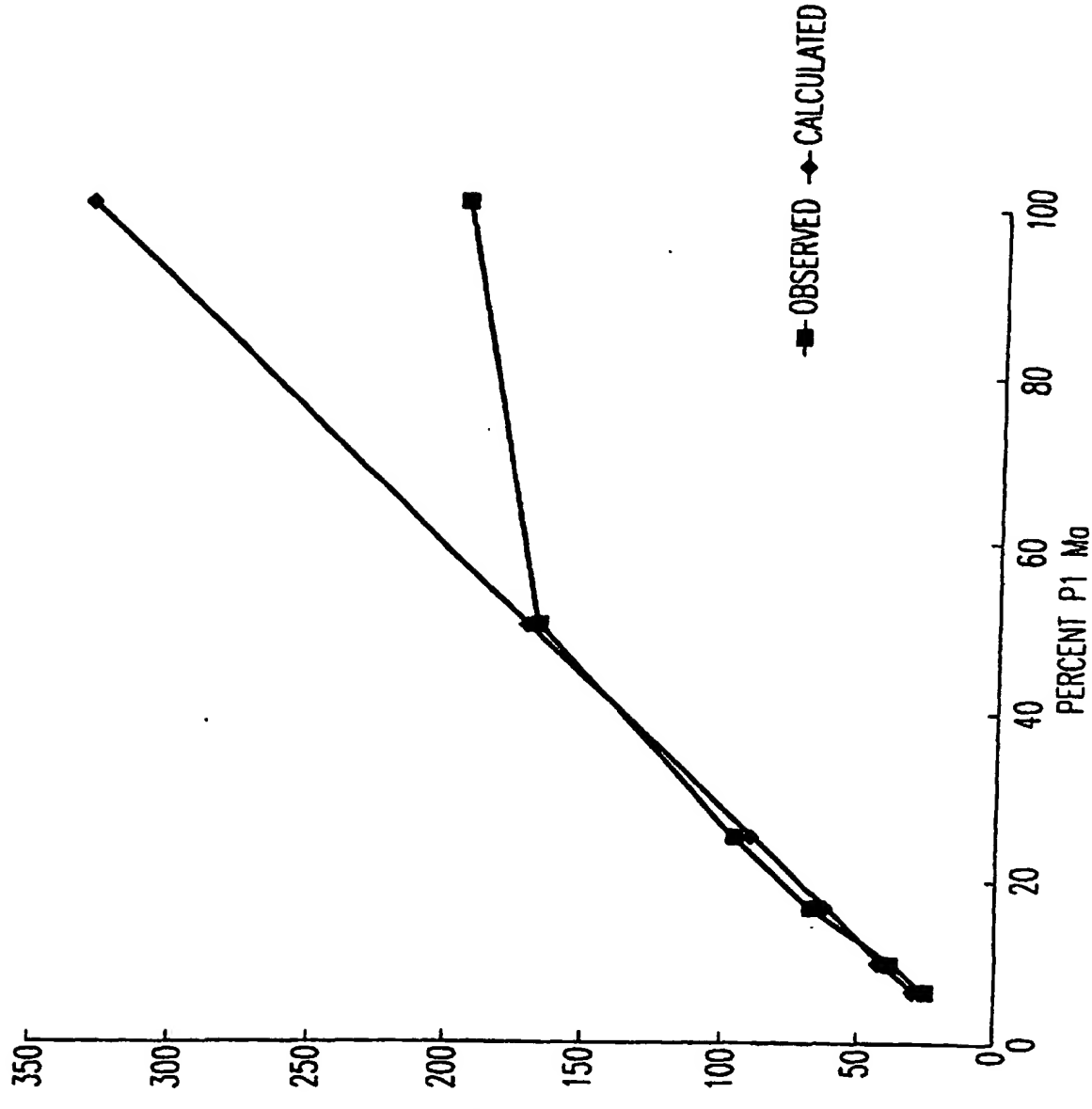

OBSERVED
CALCULATED
COUNTS BOUND
THOUSANDS
350
300
250
200
150
100
50
0
0
20
40
60
80
100
PERCENT P1 Mo

FIG. 8

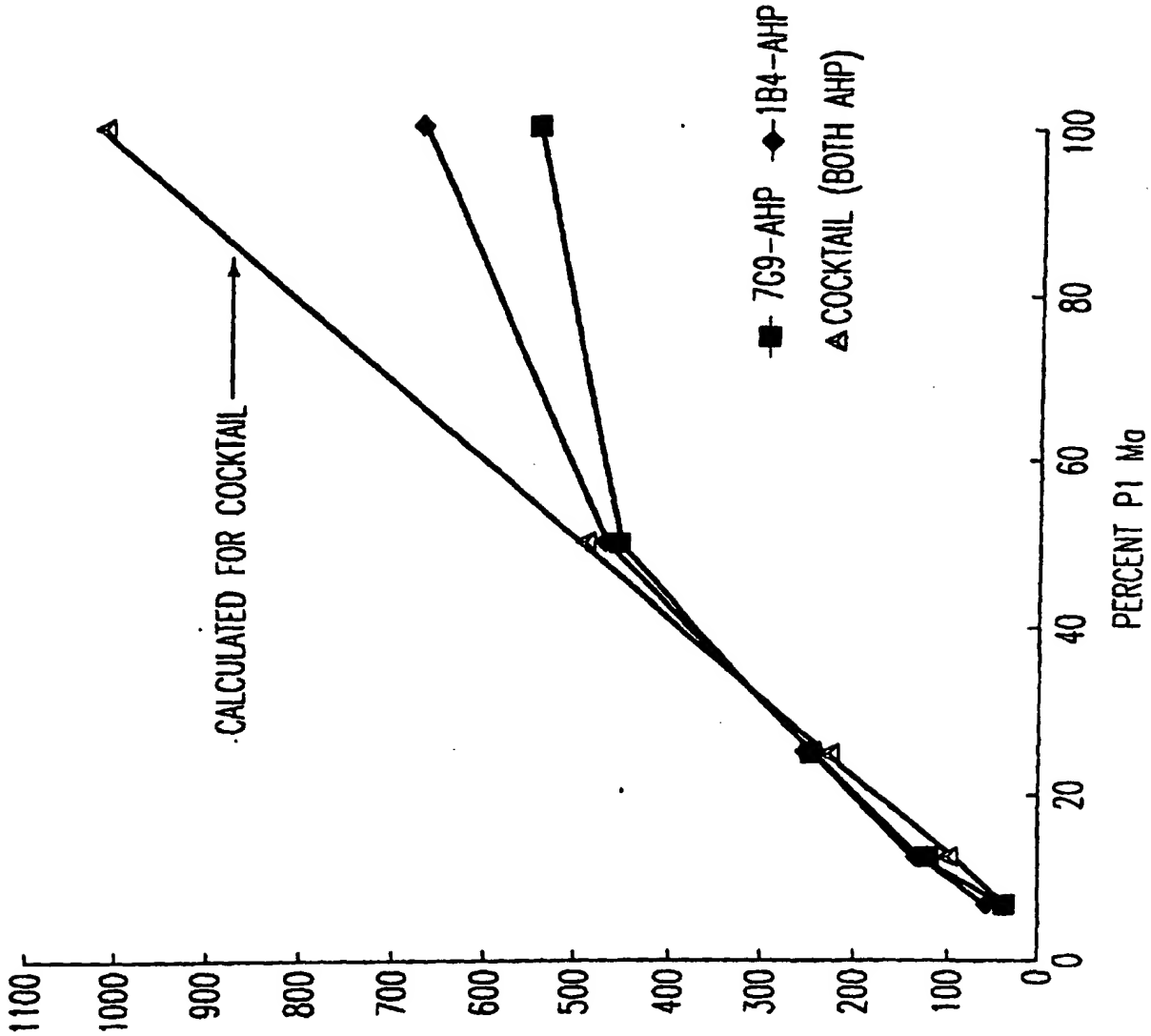

1100
1000
900
800
700
600
500
400
300
200
100
0
COUNTS BOUND
THOUSANDS
CALCULATED FOR COCKTAIL
7C9-AHP
1B4-AHP
COCKTAIL (BOTH AHP)
0
20
40
60
80
100
PERCENT P1 Mo

FIG. 9

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(54) Title: PRIMATE ERYTHROCYTE BOUND MONOCLONAL ANTIBODY HETEROPOLYMERS

(57) Abstract

The application relates to the use of antibodies in therapy. Monoclonal antibody heteropolymers are described which comprise a monoclonal antibody specific for the CR1 receptor on primate red blood cells covalently linked to a second monoclonal antibody specific for an antigen to be cleared from the circulatory system. The monoclonal antibody heteropolymers may be injected directly into a patient's circulatory system. Alternatively, red blood cells removed from a patient or a suitable donor may be contacted with the monoclonal antibody heteropolymers and then administered to the patient.

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Description

Primate Erythrocyte Bound Monoclonal Antibody Heteropolymers

Technical Field

This invention is directed to mammalian primate erythrocytes to which have been bound cross-linked monoclonal antibodies (heteropolymers) specific for both the erythrocyte complement receptor protein (CR1), and (a 2nd antibody bound thereto specific for) a circulating antigen. Methods of using these "franked" erythrocytes in diagnostic or assay methodology and therapeutic applications are also addressed.

Background Art

Mammalian primate erythrocytes (RBC's) have been identified as essential to the body's ability to clear antibody/antigen immune complexes from the blood. Specifically, the RBC receptor (CR1), known to be specific for certain activated complement proteins (C3b, C3bi and C4b), has been implicated as playing an important role in the primate's defense against microorganism infection by facilitating the neutralization and clearance of certain pathogenic substances. Other evidence shows that the binding of these immune complexes to RBC's at the CR1 site provides a vehicle for rapid clearance of potentially pathogenic immune complexes from circulation. Enhancement of phagocytosis and circulatory transport of immune complexes have both been advanced as mechanisms by which the RBC's function in this immune response have been described. See, e.g., Nelson, Science 118, 733-737 (1953) and Hebert et al, Kidney Int., 31, 877-885 (1987). In any event, defects in aspects of this RBC clearance

method have been demonstrated to be at least statistically related to a number of diseases and are believed to presage various disease activities.

Notwithstanding the importance of this function of the RBC and the immune system, it is apparent that the RBC binding and clearance capacity therefore is confined to immune complexes recognized by the CR1 receptor, that is the immune complexes must contain large amounts of at least one of the activated Complement proteins C3b, C3bi, or C4b. Thus, the mammalian primate or human body has no normal capacity to take advantage of the clearance system provided by the RBC binding ability to remove antigens not complexed with the identified activated complement proteins. It remains an object of those of skill in the art to augment the natural capacity of the mammalian circulatory system to clear antigens through RBC binding ability to include the ability to bind immune complexes (antigen/antibody complexes) via CR1 to RBC's in the absence of activated complement proteins. These augmented RBC's would be useful both in a therapeutic sense, as well as in an assay mode to identify the presence or absence of specific antigens.

Disclosure of the Invention

Specific monoclonal antibody heteropolymers are prepared from Mabs specific to the CR1 RBC receptor and Mabs to at least one other antigen and are then heteropolymerized using established techniques, as reported in U.S. Patent Application Serial No. 07/592,801 filed October 4, 1990. This heteropolymer binds readily to RBC's in whole blood, in numbers in good agreement with the number of CR1 sites available. The RBC's, if franked in vivo with the heteropolymer, will then bind

the antigen for which the remaining Mab is specific. These RBC's then can act therapeutically by facilitating the neutralization and clearance from the circulation of the bound antigen.

Alternatively, if introduced into a blood sample, franked RBC's bind quite rapidly to the antigen for which the second Mab is specific, and can also be used to assay for the presence of that particular antigen. If necessary, labeling of the heteropolymer and/or the antigen with, e.g., radioactive iodine, can facilitate bound RBC counts, and both qualitative and quantitative assessment of the antigen presence. Specifically, the heteropolymer-franked RBC's can be used in clinical assays for antigens in the circulation as demonstrated by the following example: Franked RBC's would be added to the plasma or anti-coagulated blood and allowed to bind the putative target antigen. After a wash the presence of the antigen bound to the RBC's would be revealed using appropriately labelled (e.g. either with ^{125}I, or enzyme-linked) second antibodies to the target antigen. Such assays can be either qualitative or quantitative.

RBC's removed and isolated may also be used as therapeutic agents. Once franked with heteropolymer, these RBC's can be reintroduced into the patient, where, in the bloodstream, free antigen will be bound and immobilized on the RBC, and cleared in accordance with the body's RBC clearing mechanism, which has been identified but is not completely understood. The franked RBC's can be specific for known antigens, such as HIV (the AIDS virus), or for substances which, if present in large amounts, can induce or aggravate disease states, such as low-density lipoproteins, or cause adverse biological effects, such as elevated hormone levels.

Given the general ease with which Mabs can be prepared for any known antigen, the variety of franked RBC's imaginable is unlimited. Any antigen or immunogen found in the bloodstream can be addressed by this therapeutic method.

In an alternative embodiment, RBCs are franked with a "cocktail" of several heteropolymers which, in addition to binding the target antigen, also bind to several distinct and non-overlapping sites on CR1 of the RBC. These points are identified, by e.g., Mabs 1B4, HB8592, and 57F. Experiments have demonstrated that by using two or more non-overlapping Mabs for binding to CR1 on the RBC, the number of Mab heteropolymers that can be bound to a single RBC is increased in numbers in good agreement with the number of available binding sites. This augments the capability of a relatively small number of RBC's to bind to a relatively larger amount of antigen, and can further facilitate removal of the antigen through the normal immune clearance system.

Brief Description of the Drawings

Figure 1 illustrates heteropolymer mediated binding of Human IgG to erythrocytes as compared to controls (unfilled columns in Fig. 1b). Figure 2 reflects heteropolymer mediated saturation of binding of DNP_5BGG to human erythrocytes.

Figures 3 A and B reflect <u>in vivo</u> clearance of injected antigen pursuant to the claimed invention.

Figures 4A and B gives similar data for another test individual.

Figure 5A gives similar clearance data for an independent test in another individual.

Figure 5B traces the degree of change in ^{51}Cr label throughout a clearance test.

Figures 6A, 6B and 6C reflect clearance of heteropolymers of the claimed invention from the circulation of a Rhesus monkey.

Best Mode for Carrying Out the Invention

As noted above, the flexibility of the franked RBC's of this invention in addressing a variety of disease states is limited only by the varieties of different antigens present in or accessible to the circulatory system and to which Mab can be prepared. A variety of Mab heteropolymers have been prepared. In order to attach to the RBC, the antigen-specific Mab is cross-linked with a Mab to the RBC complement receptor, CR1. Methods of cross-linking these antibodies are known to those of skill in the art. In the examples set forth below, some cross-linked heteropolymers were prepared using N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) according to established, published procedures. For details as to this procedure, see, e.g., Karpovsky et al, J. Exp. Med. 160, 1686-1701 (1984); Perez et al, Nature, 316, 354-356 (1985) or Titus et al, Journal of Immunology, 139, 3153-3158 (1987). In an alternative embodiment, heteropolymers are formed by biotinylating a Mab, incubating the biotin-bearing Mab with an avidin or strepavidin molecule, and then introducing a second Mab, also bearing a biotin linker arm. The thus-formed heteropolymer is a Mab-biotin-avidin/strepavidin-biotin-Mab sandwich. Other procedures are known to those of

ordinary skill in the art. A full listing of Mab heteropolymers prepared appears in Table 1, infra.. Prototype antigens selected for targeting through preparation of appropriate heteropolymers include dinitrophenylated bovine gamma globulin ($DNP_{55}BGG$), and human IgG. Both antigens and heteropolymers were iodinated by the IODOGEN method (Fraker et al, Biochem. Biophys. Res. Commun. 80, 849-857 (1978). Iodination provides one protocol for assay utilization, but of course need not be practiced for the therapeutic aspects of the claimed invention.

Assays for CR1 levels on isolated RBC's followed standard methods, revealing about 200-500 epitopes per RBC, as bound to by anti-CR1 Mabs 1B4, 3D9 and HB8592.

Details regarding RBC binding, binding kinetics and observed values follow below.

Examples:

1) Direct sensitization and binding isotherm analyses: Between 0.1 and 1.0 ml of a 10%-50% dispersion of washed RBC's in bovine serum albumin/phosphate buffered saline (BSA-PBS) were reacted for 1 hr at room temperature, with shaking, with varying amounts (10-50 ul) of a dilution of one or more of the heteropolymers. The RBC's were then washed 3 times in BSA-PBS (to remove excess unbound heteropolymer) and, after reconstitution in either BSA-PBS or normal human serum (for experiments with ^{125}I-human IgG and $DNP_{55}BGG$, respectively), mixed with a small volume of ^{125}I-probe. After a further incubation (usually 1 hr at room temperature, with shaking), RBC-bound and free ^{125}I-antigens were separated by either of two procedures: RBC's were spun through oil

(typically 150 ul of reaction mixture was layered on 200 ul of a dibutyl-dinonyl phthalate mixture), or simply processed by two cycles of centrifugation and washing in BSA-PBS. RBC-associated ^{125}I counts were quantitated in a Beckman 5500 gamma counter.

2) "Whole Blood" binding kinetics: a) In most procedures blood was drawn into Alsever's and centrifuged. A portion of the supernatant was removed, and after the blood cells were redispersed to a final hematocrit of 50%, a small amount of ^{125}I-DNP_{55}BGG antigen was added. Varying amounts of heteropolymer were added directly to aliquots of these "whole blood" dispersions containing ^{125}I-DNP_{55},BGG, and incubated with shaking at 37°C. RBC associated ^{125}I counts were determined at varying time points after centrifugation and washing steps. Selected aliquots of the reaction mixtures were also centrifuged through percoll to confirm that only RBC's (not white cells) bound the ^{125}I-antigen. In some of these "whole blood" experiments, instead of using Alsever's as an anti-coagulant, blood was drawn into EDTA or citrate and used at once in a similar manner. A few comparable "whole blood" experiments were also performed with ^{125}I-human IgG as the target antigen. In these experiments washed RBC's were dispersed in BSA-PBS, to avoid the potential confounding effect of endogenous serum-associated IgG. b) In other kinetic experiments one volume of RBC's was franked with saturating amounts of the heteropolymer, and after three washes was added to 10 volumes of anti-coagulated blood containing ^{125}I-DNP_{55}BGG, and incubated at 37°C. Aliquots of the dispersions were processed periodically to determine RBC-associated ^{125}I counts.

Direct binding of ^{125}I-heteropolymers to a number of

matrices was determined in procedures analogous to those described above. For example, duplicate aliquots of 100 ul of ^{125}I-heteropolymer #4 (see below) were incubated for one hour at room temperature, with shaking, with either 100 ul of a 50% dispersion of human RBC's, or 100 ul of a 33% dispersion of human IgG-Sepharose. Samples were then subjected to two cycles of centrifugation and washing and the levels of matrix-bound ^{125}I counts were determined. Direct binding to human RBC's of the ^{125}I-heteropolymers was also determined as a function of time at 37°C.

Control experiments tested for the specificity of antigen binding by heteropolymer treated RBC's and verified the requirement for CR1. These experiments included the use of heteropolymer-treated sheep RBC's (which lack CR1), naive (untreated) human RBC's, and excess homologous monomeric Mabs (in ascites fluid) which blocked the action of the heteropolymers.

Results

Preparation and Initial Characterizations of Heteropolymers. We prepared a number of heteropolymers by SPDP cross-linking, and examined the ability of these heteropolymers to react with human RBC's and facilitate binding of specific antigens. Preliminary data (Table 1), using mixtures of saturating amounts of unfractionated material (containing heteropolymers and non-cross-linked monomers), demonstrated specific RBC-associated binding of the ^{125}I-antigens. An excess of ^{125}I-antigen was used in order to determine the maximum number of ligands bound per RBC. For each heteropolymer mixture the results (Table 1) are in good agreement with the typical number of CR1 epitopes (200-500) recognized by the anti-CR1 Mabs.

Heteropolymer mixture #1 can facilitate binding via two noncompeting Mabs to CR1, 1B4 and HB8592. This mixture can, therefore, place approximately twice as many anti-IgG heteropolymers on the RBC's as a heteropolymer containing only one anti-CR1 Mab. The maximum ^{125}I-human IgG bound to such "doubly-franked" RBC's is nearly equal to the sum of the ^{125}I-IgG bound to RBC's franked with two individual components of the mixture (Table 1); this illustrates the principle of additivity. Dose-response experiments with heteropolymer #1 and other heteropolymers (Table 1, and see below) confirm that RBC binding of both heteropolymer and ^{125}I-antigen is saturable. "Background" binding of antigen to naive RBC's is low, and use of heteropolymers with "irrelevant" specificities for the target ligands (e.g. 8E11 (anti-C3b) X HB8592) gave no binding (Table 1).

Binding Isotherms with Isolated Heteropolymers. Heteropolymer mixtures were further purified by gel permeation chromatography, and the highest mw subfractions (ca. corresponding to trimers and larger species) were used to quantitate binding (Figures 1 and 2). In these experiments binding of ^{125}I-antigens to franked RBC's was determined, after two cycles of centrifugation and washing with BSA-PBS, by direct counting of the RBC pellets.

At saturating input of heteropolymer, the maximum number of antigen molecules bound per RBC is in good quantitative agreement with our initial survey results using unfractionated heteropolymer mixtures and centrifugation through oil to separate bound from free ^{125}I-antigen. These experiments confirm that binding is saturable, since use of excess quantities of a single heteropolymer or ^{125}I-antigen does not increase binding

beyond the saturation level (typically 200-1000 antigens per RBC, Figures 1 and 2). Analysis of results with blood from two donors (Figure 1a and 1b) demonstrates that maximum binding reflects the number of CR1 epitopes per RBC characteristic of the individual donor. The principle of additivity is also illustrated in experiments in which RBC's were franked with a combined mixture of two heteropolymers (Figure 1a). The combined action of the two heteropolymers in facilitating binding of the ^{125}I-antigen is close to the sum of the action of each species individually.

Bi-specificity of the heteropolymers was demonstrated in inhibition experiments using an excess of homologous monomeric Mab. Our goal was either to block binding of heteropolymer to RBC's (using an appropriate anti-CR1 Mab to thus preclude binding of ^{125}I-DNP$_{55}$BGG), or to inhibit directly binding of ^{125}I-DNP$_{55}$BGG to franked RBC's (using the appropriate monomeric anti-DNP Mab). In all cases more than 90% of specific binding was reduced by these procedures (Figure 1b). Sheep RBC's lack CR1, and as anticipated, heteropolymers directed against CR1 do not facilitate binding of the ^{125}I-antigen to sheep RBC's (Figure 1b). Finally, the dual specificities of two of the heteropolymer mixtures was confirmed by labelling them with ^{125}I and examining their binding to human RBC's and to a Sepharose 4B matrix containing their respective target antigens (Table 2). The results demonstrate that the isolated polymers bind to both of their respective matrices, and also confirm that their direct binding to human RBC's is rapid at 37°C.

TABLE 1

Survey of Cross-Linked Mab Heteropolymer Mixtures in facilitating Antigen Binding to RBC's

A. Binding of ^{125}I-Human IgG

Hetero Polymer #	Mab #1 (Specificity) X Mab #2 (Specificity) *	Molecules** IGG Bound/ RBC
1	1B4(anti-CR1) X HB43 (anti-IgG) and HB8592 (anti-CR1)	1150
2	HB43(anti-IgG) X HB8592 (anti-CR1)	601
3	HB43(anti-IgG) X 1B4(anti-CRI)	492
4	HB43(anti-IgG) X HB8592(anti-CR1)	357
5	HB43(anti-IgG) X 1B4(anti-CR1)	479
6	HB43(anti-IgG) X 3D9(anti-CR1)	355
7	HB43(anti-IgG) X 57F(anti-CR1)	387
Control	8E11(anti-C3b) X HB8592(anti-CR1)	-2

B. Binding of ^{125}I-$(DNP)_{55}BGG$

$DNP_{55}BGG$ Heteropolymer	Mab #1 (Specificity) X Mab #2 (Specificity) *	Molecules Bound/ RBC
8	3D9(anti-CR1) X 2A1(anti-DNP)	191
9	3D9(anti-CR1) X 23D(anti-CR1)	243
10	23D1(anti-DNP) X HB8592(anti-CR1)	129
11	23D1(anti-DNP) X 1B4(anti-CR1)	255
12	23D1(anti-DNP) X 3D9(anti-CR1)	196
13	HB8592(anti-CR) X 2A1(anti-DNP)	95
14	HB8592(anti-CR1) X 23D1(anti-DNP)	133
15	1B4(anti-CR1) X 23D1(anti-DNP)	279
16	1B4(anti-CR1) X 2A1(anti-DNP)	236
Control	8E11(anti-C3b) x HB8592(anti-CR1)	-11

C. Demonstration of Saturation of Binding with Heteropolymer #1 and ^{125}I-IgG

Relative Heteropolymer Concentration	Relative ^{125}I-Human IgG Concentration	Molecules** IgG Bound RBC
5	5	994
5	1	868
5	0.2	343
1 ***	1 ****	777
0.2	1	205

Table 1 footnotes

* For each heteropolymer listed, the first Mab was reduced with dithiothreitol (after reacting with SPDP) and then coupled to the second SPDP-reacted Mab. Heteropolymers #2 and #4 represent preparations with HB8592 purified via protein G and octanoic acid-50% saturated ammonium sulfate, respectively. Heteropolymer # 1 was prepared by simultaneously reacting a cocktail of SPDP-coupled and reduced 1B4 and HB8592 with SPDP-coupled HB43.

** Binding was determined by centrifuging RBC's through oil. Background binding to naive human RBC's was 40 human IgG and 60 $DNP_{55}BGG$ per RBC respectively, and was subtracted to give the net specific binding reported. In parts A and B predetermined saturating inputs of both heteropolymer and ^{125}I-antigen were used.

*** "1" corresponds to 3.0 ug/ml of heteropolymer in a 12.5% hematocrit.

**** "1" corresponds to 0.92 ug/ml ^{125}I-human IgG in a 12.5% hematocrit.

TABLE 2

Binding of ^{125}I-Labelled Heteropolymers to Human RBC's or to Sepharose Coupled Ligands

	% Bound *	
	Human RBC's	IgG (or DNP)-Sepharose
#4, unfractionated mixture (HB43(anti-IgG) X HB8592(anti-CR1))	45 ± 5	55 ± 5
#4, isolated polymer fraction	65 ± 3	86 ± 5
#11, unfractionated mixture (23D1(anti-DNP) X 1B4 (anti-CR1))	34 ± 6 **	86 ± 5
#11, isolated polymer fraction	72 ± 2 ***	85 ± 5

Table 2 footnotes

* Bound after incubation (with an excess of binding matrix) for one hour at either room temperature (Sepharose samples) and/or 37 °C (RBC's were examined at both temperatures). IgG Sepharose was used as the binding matrix for heteropolymer #4, and DNP-Sepharose (containing a dinitrophenylated Mab to IgM) was used for #11. All samples were corrected for background binding (5% or less) to sheep RBC's or naive (unreacted) Sepharose.

** Binding was 30% and 32% respectively after incubation for either two or five minutes at 37°C.

*** Binding was 57% and 69%, respectively, after incubation for either two minutes or five minutes at 37°C.

PRIMATE STUDIES:

Materials and Methods

Mab and Heteropolymer Preparation. Purified Mab to CR1, the DNP group, and human IgG were biotinylated following published procedures Wilchek et al, Meth. Enzym., 184, Avidin-Biotin Technology (1990) using the "long-arm" biotinylating agent biotinyl-N-hydroxy succinimide (Vector Laboratories, Burlingame, CA) at molar inputs of biotin to Mab between 5 to 1 and 20 to 1. The Iodogen method Fraker et al, Bioc & Biop. Review, 80:849 (1978) was used to label both naive and biotinylated Mab with ^{125}I. DNP_5BGG Little et al, Neth. Immun. Immunochem, 1:128 (1967), was similarly labelled with either ^{125}I or ^{131}I. Preparation of soluble cross-linked heteropolymer (HP) which specifically bound to primate E was accomplished by first incubating (at 37°C for 30 min) a biotinylated Mab to CR1 (either $1B4_{10}$ or $E11_{10}$) with excess strepavidin (SA) (Gibco BRI, Gaithersburg, MD). Subsequently a biotinylated Mab to the DNP group ($23D1_5$) was incubated with this complex, and the resultant HP was then used without further purification. Detailed dose response tests were performed to determine the optimum inputs of biotinylated first Mab (anti-CR1), SA, and biotinylated second Mab (antiDNP) in the generation of soluble HP which bound to human and other primate erythrocyte (E) and facilitated antigen (Ag) binding. In all cases we verified that the HP used in these studies neither directly bound to, nor facilitated Ag binding to sheep E, which lack CR1 (22).

RIA. Varying inputs of radiolabelled HP (either the anti-CR1 Mab or anti-DNP Mab portion of the HP was radiolabelled) were incubated at 37°C for 5-15 min with a

50% dispersion (in homologous serum) of human or primate E [a "whole blood" simulation Ross et al, J. Immunol., 135:2005 (1985)]. Binding was determined by counting the E pellets after centrifugation and washing. To quantitate HP-mediated binding of Ag (DNP_5BGG) to E, the radiolabelled Ag was first added to the "whole blood" dispersion, and after varying amounts of HP were added, the samples were processed similarly. In some in vitro Ag-binding experiments freshly drawn anti-coagulated blood was used, and Ag binding via the HP was also demonstrable.

The number of CR1 epitopes per E was determined by incubating an excess of ^{125}I-labelled Mab to CR1 with isolated and washed E followed by one of two procedures for separation of E-"bound" from "free" Mab: 1) the incubation mixtures were layered on dibutyldinonyl phthalate mixtures, and after a centrifugation step the samples were frozen and the E pellets were cut off or counted; 2) alteratively, the E dispersions were simply pelleted, and after washing, the E pellets were counted. In all RIA, sheep E controls were included to provide a background correction, which was less than 10% of specific binding to human E.

In Vivo Experiments. We followed protocols similar to those we previously reported for studying clearance of IC from the circulation of both non-primates and primates. See, e.g., Taylor et al, J. Immunol. 139:1240 (1987). The doses of Ag and HP chosen in these experiments were optimized based on titration experiments performed in anti-coagulated whole blood samples from both humans and monkeys: 1 ml of radiolabelled DNP_5BGG (3-25 μg) in PBS was infused within 30 seconds into the catheterized saphenous vein of a 1 kg squirrel monkey

sedated with ketamine and maintained under anesthesia with halothane. After 20-35 min, 1 ml of HP (0.25-1.0 mg, either radiolabelled or unlabelled) was injected. Multiple blood aliquots (anti-coagulated in citrate) were collected over the entire course of the experiment to determine both the "endogenous" clearance rate of the Ag, and the effect of the HP on its clearance and E binding. Within 10 min of collection each blood sample was centrifuged and the cell pellet washed and counted to determine binding. The supernatants were combined and counted, and aliquots were examined to determine net 5% TCA-precipitable counts. Initially after injection, ^{125}I and ^{131}I counts recovered in the plasma supernatants were > 95% and 85% TCA-precipitable, respectively. Finally, selected blood samples were centrifuged through percoll gradients (which only allowed pelleting of the E). This procedure verified that cell-associated counts were only due to E binding. The same basic methodology was followed in our experiments with 10 kg rhesus monkeys, except 0.25 mg of HP was injected.

Quantitative Analyses. The specific activities of all proteins were determined and used to calculate the number of molecules bound per E for both *in vivo* and *in vitro* experiments. In the case of double or triple label experiments (^{125}I and ^{131}I, or both iodine labels and ^{51}Cr) the overlap of different labels was determined in calibration experiments and corrected counts were calculated for each isotope.

Assays for an Immune Response. We used a solid phase "Ag capture" assay, Khazaeli et al, J. Biol Resp. Med., 91:178 (1990) to determine if the animals developed an immune response to the injected agents (biotinylated mouse IgG, SA, and DNP_5BGG). This assay protocol

involves adhering the Ag to a solid matrix, adding a 10-20 fold dilution of the serum to be tested, and then adding radiolabelled Ag as a probe. Specific binding of antibody to solid phase Ag then allows capture of solution phase radiolabelled Ag. Positive controls in this assay included goat anti-mouse IgG, biotinylated human IgG, and mouse anti-DNP Mab (23D1), respectively. Alternatively, in order to increase the sensitivity of the assay, we "developed" with ^{125}I-Mab HB43, an anti-human IgG Mab which cross reacts with monkey IgG. This "direct" solid phase RIA, is considerably more sensitive for detecting a weak immune response (see below).

Results

In vitro preliminary studies in squirrel monkeys. We focused on the use of anti-CR1 Mab 1B4 in these clearance studies because it is known to block the "active site" (the C3b-binding site) of human CR1 (30) and therefore should bind close to the most "biologically relevant" site in terms of in vivo recognition by fixed receptors on liver or spleen cells. Also, large amounts of this Mab can be prepared from the hybridoma cell line. However, even though there are a moderate number of CR1 epitopes on the squirrel monkeys' E, defined by Mab E11, there are far fewer binding sites for Mab 1B4 on the monkey E compared to the number found on human E (Table 3A). Binding of Mab 1B4 to squirrel monkey E also appears to be of lower avidity than binding to human E, as evidenced by the relatively large reduction in net binding to the monkey E when the E are processed through a wash step, rather than simply by centrifugation through oil (Table 3A).

Construction of soluble, cross-linked multivalent A/B HP with biotinylated Mab $1B4_{10}$ considerably enhanced binding of this Mab to squirrel monkey E and caused an increase in binding to human E as well (Table 3B). Use of a radiolabel on the "second" (anti-target Ag) biotinylated Mab $23D1_5$ of the HP confirms that the A/B system does fix both biotinylated Mab to the primate E (Table 3B). The specificity of binding is confirmed by several experiments: "Background" binding of the HP to sheep E (lacking CR1) is much lower; and, in the presence of excess monomeric Mab 1B4 (in ascites), HP-mediated binding to human and squirrel monkey E is almost completely eliminated (Table 3C). The results also indicate that more HP-associated, biotinylated $23D1_5$, Mab is bound to the E than the biotinylated $1B4_{10}$ Mab, which is probably a consequence of amplification due to the multivalent nature of the A/B system. Finally, binding of the biotinylated $23D1_5$ Mab to primate E is reduced to background levels (the level seen for sheep E) unless both biotinylated $1B4_{10}$ and SA are used for construction of the HP (data not shown).

We used A/B HP prepared with biotinylated preparations of Mab 23D1 and either Mab 1B4 or Mab E11, to facilitate binding of the target Ag DNP_5BGG to primate E (Table 4) in a "whole blood" experiment analogous to those we have reported using SPDP-HP. It can be seen that comparable binding is obtained for HP constructed with either Mab $E11_{10}$ or $1B4_{10}$. We chose DNP_5BGG (a protein with a low degree of dinitrophenylation) as binding substrate for the in vivo experiments because it has been reported that a high degree of protein dinitrophenylation leads to rapid clearance from the circulation, even in the absence of specific antibody. The affinity of Mab 23D1 for the DNP group is only ca.

$5x10^6$ L/M, which is consistent with the moderate degree of binding (20-40%) of the DNP_5BGG by E opsonized with a HP containing $23Dl_5$. A similar level of target Ag binding is demonstrable in vivo (see below).

In vitro preliminary studies in cynomolgus and rhesus monkeys. The level of detectable E11 and 1B4 epitopes on the E of these primates was higher than that detected for the squirrel monkeys (Table 5). In these experiments we prepared A/B HP using a "second" Mab, HB43, specific for human IgG. In vitro binding of these HP to monkey E is quantitated in Table 5.

In vivo clearance kinetics in squirrel monkeys. Injection of ^{125}I-DNP_5BGG into the squirrel monkey leads to rapid clearance of a fraction of the injected Ag counts within the first 15 min, followed by a slower phase of clearance (Figure 3). After 20 min we injected a $1B4_{10}/SA/23Dl_5$ HP specific for both monkey CR1 and the DNP group. Within 10 min ca. 20% of the circulating Ag counts became E bound. Consistent with the uptake of counts by the E is a comparable loss of counts from the plasma. It is also evident that the majority of E-associated counts are then cleared rapidly from the circulation because a significant number of these counts do not return to the plasma phase. Finally, those plasma counts that did not become E-bound continued to be cleared at a relatively slow rate.

Clearance of both target Ag and HP were then followed through a double label experiment. The data in Figure 4A again demonstrates a rapid initial phase of Ag clearance followed by an approximate plateau and subsequent slow removal of plasma counts from the circulation. It should also be noted that less than 3%

of the counts are E-bound before the HP is injected (the first 25 min of the experiment). Upon injection of HP there is a drop in Ag-associated plasma counts which is ca. equal to the generation of E-associated counts (a maximum of 31% of the Ag was bound to the E). It is also evident that the majority of injected HP binds rapidly to the E (a maximum of 60%), and that subsequently both E-bound HP and Ag are cleared at approximately the same rate. The co-clearance of E-bound HP and Ag is also illustrated in a comparable experiment performed on a different squirrel monkey (Figure 4B). We also conducted an independent double label experiment similar to that depicted in Figure 4A, except the ^{125}I label was on the $23Dl_5$ Mab (Figure 5A). The rate of clearance of E-bound HP and Ag was again approximately the same, and although maximum binding of Ag was 35% (comparable to earlier experiments), the maximum level of HP binding (defined by the ^{125}I label on the anti-DNP $23Dl_5$ Mab) was reduced to 40%.

In order to address the potential problem associated with HP-mediated lysis of the E we co-injected ^{51}Cr labelled E along with ^{131}I-labelled DNP_5BGG, and followed both labels. After 35 min ^{125}I-labelled HP was injected and the clearance of all 3 radiolabels was monitored. Clearance of the ^{131} I- labelled DNP_5BGG (ca. 25% maximum E binding) followed the same trends seen in previous experiments (data not shown). Throughout the course of the experiment the ^{51}Cr label remained associated with the E pellet, and showed no significant change before or after injection of the HP (Figure 5B). In this experiment, 50% of the ^{125}I-labelled HP bound to the E, and the E-associated counts were cleared rapidly. Also, in agreement with the results shown in Figure 2, the fraction of HP that remained in the plasma was cleared at

a much slower rate.

In vivo clearance kinetics in rhesus monkeys. The level of HP binding (per E) to the squirrel monkey E was much higher than the level of Ag binding, and it is unlikely the Ag itself facilitated clearance of the majority of E-bound HP. However, it is important to demonstrate that the present results can be generalized to other primates and other Ag systems. For these reasons we used the A/B system to prepare HP with biotinylated Mab $1B4_{10}$ and human IgG_5, or with biotinylated Mab $E11_{10}$ and $HB43_5$ (an anti-human IgG Mab), and injected these HP into rhesus monkeys (Figures 6A and 6B). In these experiments we followed the HP with a radiolabel on the "second" antibody, rather than on the anti-CR1 Mab. The results clearly indicate that both these HP bind to the primate E and are then cleared rapidly. Once again the fraction of HP that did not bind to the E and remained in the plasma was cleared at a much slower rate. Consecutive experiments were performed on the same animals by injecting a second HP one hr after the first HP (Figure 6C). The overall clearance of the second HP from the animal's circulation was similar to that seen for the first injected HP.

We measured CR1 on E of the rhesus monkeys from blood aliquots obtained before and during these experiments, and the level remained constant (approximately equal to the levels reported in Table 5) during the course of the procedure. Finally, we tested these HP for complement activation and C3b capture after binding to human E in homologous serum. In some instances there was a low level of C3b binding to the E, but in other cases (e.g., the 1B4/human IgG HP) we could not detect any C3b bound to the E above background

levels.

In preliminary experiments (performed 3 weeks prior to those discussed above) we injected rhesus monkeys with a "partial HP" containing radiolabelled-biotinylated anti-CR1 Mab and SA only (lacking the "second" biotinylated antibody) in order to determine if this "partial" HP could bind E and be cleared from circulation. Although binding of this complex to E was approximately the same as that of a complete HP ($1B4_{10}$/SA/$23D1_5$), no significant short term clearance was observed, but after 3 weeks all counts had been cleared from circulation.

Development of an immune response. We conducted a total of 3 similar experiments on each of 2 squirrel monkeys over a period of 6 weeks. In order to determine if the squirrel monkeys developed an immune response to any of the injected materials (biotinylated mouse Mab, SA, or DNP_5BGG) we performed solid phase "Ag capture" assays on serum samples taken from the animals before, during, and after the experiments. The results of this assay indicate that there was no demonstrable immune response in any of the squirrel monkeys over a period of 10 weeks. However, by assaying with the more sensitive "direct" solid phase RIA (see Materials and Methods), we found that squirrel monkey 417 (but not 899) exhibited a rather weak, but demonstrable immune response to mouse IgG (but not SA or DNP_5BGG). The immune response was detected after the first experiment and increased slightly with time. Dose response experiments using the "direct" RIA indicate the binding capacity of a 5-fold dilution of serum from squirrel monkey 417 was comparable to that of a 10,000 fold dilution of goat antimouse IgG.

Two clearance experiments were performed on the rhesus monkeys, with a 3 week interval between experiments (see above). The "Ag capture" assay revealed no immune response to biotinviated mouse IgG during this time period. However, there was weak but measurable binding of mouse IgG by sera from both rhesus monkeys 2 weeks after the second experiment (data not shown). Using the more sensitive "direct" solid phase RIA (probing with ^{125}I-HB43) indicated the rhesus monkeys had developed a weak but demonstrable immune response to biotinylated mouse IgG by the start of the second clearance experiment. The titer of anti-mouse antibodies peaked 2 weeks after the second experiment and persisted in the circulation for more than 2 months. At the peak level (day 36), dose response experiments indicated that binding of the ^{125}I-HB43 probe corresponded to a titer of approximately 0.5% of that of a positive control, goat anti-mouse IgG (Table 6).

Table 3. Binding of anti-CR1 Mab and HP to Squirrel Monkey and Human E.

A. Direct CR1 Measurements[a]

		Molecules Bound per E[b]				
mAb	Binding Assay	S. Monkey 899	S. Monkey 417	S. Monkey 413	Human 1	Human 2
E11	Oil	310	280	290	578	510
E11	Washed Pellet	210	210	230	480	410
1B4	Oil	50	30	50	340	330
1B4	Washed Pellet	10	5	20	280	260
$1B4_{10}$[c]	Oil	55	30	60	310	310
$1B4_{10}$	Washed Pellet	10	4	20	240	230

B. "Whole Blood" Solution Phase Binding of HP[a,d]

	Molecules Bound per E[b]				
HP	S. Monkey 899	S. Monkey 417	S. Monkey 413	Human 1	Human 2
^{125}I-$1B4_{10}$/SA/$23D1_5$	640	470	600	1090	780
$1B4_{10}$/SA/^{125}I-$23D1_5$	4700	2330	5220	7460	4660

C. Inhibition of "Whole Blood" Solution Phase Binding by 1B4 Ascites

		Molecules Bound per E[a,e]			
HP	Inhibitor	S. Monkey 417	S. Monkey 413	Human 1	Human
^{125}I-$1B4_{10}$/SA/$23D1_5$	----	430	350	510	1000
^{125}I-$1B4_{10}$/SA$23D1_5$	1B4 Ascites[f]	54	43	60	-5

Footnotes for Table 3

a) The uncertainties in the reported values average ± 5%. In all cases controls with sheet E (which lack CR1) were used to subtract the background baseline level due to nonspecific binding.

b) E were separated from excess iodinated Mab by either spinning through an oil cushion or by pelleting and washing the E.

c) The subscripts refer to the molar input of biotin to Mab in the biotinylation reaction.

d) HP were assembled in solution and added to a dispersion of E in serum containing 0.01M EDTA, and bound molecules (Mab) were determined by counting the washed E pellets.

e) Half as much HP was incubated per E in part C compared to part B.

f) 1B4 ascites fluid was pre-incubated with the E in part C. Irrelevant ascites from an anti-C3b Mab gave the same results as the sample lacking inhibitor.

Table 4. HP-mediated Binding ("Whole Blood" Solution Phase Assay) of ^{125}I-DNP_5BGG to Squirrel Monkey and Human E.[a]

HP	% Bound				
	S. Monkey 899	S. Monkey 417	S. Monkey 413	Human	Sheep
$E11_{10}/SA/23D1_5$	28	38	20	34	1
$1B4_{10}/SA/23D1_5$	42	44	14	38	1
(Naive E)	2	2	2	1	1

Molar impact of $^{125}DNP_5BGG$ corresponded to 37 molecules per E.

Table 5. Binding of Anti-CR1 Mab and HP to Rhesus Monkey and Cynomolgus Monkey E.

	Molecules Bound Per E[a]		
Mab[b]	Rh Monkey 4F	Rh. Monkey 941	Cy Monkey 69
E11	1760	1580	1480
1B4	230	140	110
HP[c]			
$1B4_{10}$/SA/^{125}I-$HB43_5$	710	700	480
$E11_{10}$/SA/^{125}I-$HB43_5$	620	610	460

a) See footnote a, Table 3.

b) Binding was determined by centrifugation through oil.

c) A relatively low input of HP per E was used. Binding values in a comparable experiment for an E Sample from a human were 1330 and 810 molecules per E for the HP containing $1B4_{10}$ and $E11_{10}$, respectively.

Table 6. Representative "Direct" Solid Phase RIA for Detecting an Immune Response to Biotinylated Mouse IgG in Rhesus Monkey 941.

A. Binding as a Function of Time[a]

	% ^{125}I-HB43 Bound to Solid Phase Matrix	
Serum Source[b]	$1B4_{20}$[c]	Mouse IgG[c]
pre-immune serum	0.8	0.8
Day 21	6.9	6.1
Day 36	9.5	15.3
Day 49	4.7	8.0
Day 80	1.9	2.9
goat anti-mouse IgG (Control)	23.2	22.2

B. Relative Titer (Day 36)

	% ^{125}I-HB43 Bound to Solid Phase Matrix[d]	
Serum and Dilution	$1B4_{20}$[c]	Mouse IgG[c]
Serum/20	12.2	15.6
Serum/80	4.0	4.2
goat anti-mouse IgG/200	36.5	37.0
goat anti-mouse IgG/1000	21.5	30.0
goat anti-mouse IgG/5000	4.6	9.4

a) The first injection (of the "partial" HP) was on day 1, and on day 21 the "complete" HP was injected.
b) All sera were examined at a 10 fold dilution.
c) Either $1B4_{20}$ or mouse IgG was used to first coat the plates (see Materials and Methods).
d) A different input of ^{125}I-HB43 was used in Part B compared to that in Part A.

METHODS OF USE

The franked RBC's described above have immediate application in a variety of research, clinical diagnostic, or therapeutic uses. The most important are therapeutic uses, which can include (1) using a franked RBC of the invention with specificity to an antigen such as HIV to clear free antigen from the blood of a human or primate patient, (2) using a franked erythrocyte with a specific Mab to clearfor a non-immunogenic but potentially "pathogenic" target such as LDL which has been linked to atherosclerosis and (3) using a franked erythrocyte with Mab specificity for the natural ligand of CR1 (such as C3b) where the number of naturally occurring receptors in an individual patient has decreased, such as in systemic lupus erythematosus. Of course, the specific antigens or antibody targets identified above are exemplary only, and virtually any circulating microorganism, virus, compound and the like to which a Mab can be prepared can be subject to therapeutic treatment through the invention.

The franked erythrocytes may be prepared and introduced for therapeutic use in either of three methods. First, the bi-specific heteropolymer comprised of at least two cross-linked Mab, one specific for the RBC and the other for the antigen, may be introduced directly to the bloodstream through inoculation. Alternatively, a small amount of RBC's can be extracted from the patient, and bound to the heteropolymer in sterile <u>in vitro</u> conditions and then reintroduced into the patient. Finally, in cases of low CR1 or low RBC disease states, franked erythrocytes from a compatable heterologous matched blood donor can be used. In any of the above examples, a "cocktail" of several

heteropolymers (see results section) can be used. Given the high binding capacity of the heteropolymer to the RBC, direct injection of the heteropolymer can be as effective as in vitro preparation of the franked erythrocyte, followed by inoculation.

In addition to taking advantage of the body's natural defenses by augmenting the natural immune defense system, binding the heteropolymer to the RBC's (either in vitro or in vivo) may reduce or remove any "immunogenicity" that would be characteristic of Mabs prepared from mouse hosts and the like. In fact, currently, the vast majority of monoclonal antibodies are produced in mice. Mab treatment thus suffers, at least to some degree, from the body's natural immune response against the mouse Mab which would thus prevent the Mab from binding to its target antigen. As the number of heteropolymers bound per RBC is relatively small (below about 500) the Mab itself may not be recognized as foreign, and the host immune response may not be triggered, or at least, will be significantly reduced. This appears to be born out by the results reported. Use of available human Mabs to prepare heteropolymers should also eliminate any host immune response.

The dosage and treatment regimen will vary from antigen to antigen, individual to individual, and disease state. In general, these can be determined on an empirical basis. An extreme minority of available RBC's may be used effectively in conferring therapeutic treatment. This is due in part to the vast numbers of RBC's present in the blood, in contrast to most antigens. As an example, the level of HIV which circulates free in the blood has been suggested as the most cytopathic form of HIV. High levels of HIV in the circulation appear to

correlate with disease activity. Yet, this level ranges between 1,000 and 50,000 virus particles per ml. Ho et al, New England Journal of Medicine, 321, 1621-1625 (1989), Coombs et al., ibid. 1626-1631. In contrast, the number of RBC per ml in circulation is many orders of magnitude greater, and accordingly, even a small minority of available RBC's treated according to the claimed invention should be sufficient to confer therapeutic treatment, given the appropriate anti-CR1/anti-HIV franked RBC. As exemplary levels only, in the treatment of AIDS, an intravenous administration of no more than 1-4 mg of appropriate heteropolymer should be sufficient to frank the patients' RBC's for quantitative binding of circulating HIV. With sufficiently high avidity anti-HIV antibodies in the heteropolymer (easily achieved by standard methods) it should be possible to use considerably less heteropolymer (μg amounts). See the " Detailed Calculations Section" for a more complete analysis of this problem. Alternatively, if the RBC's of the patient are first removed and franked with heteropolymer and then re-injected, the dose administration of franked erythrocytes would be considerably less than 1 "unit" (1 pint) of blood. Use of ca. 50-100 ml of franked RBC's (a few % of total circulating RBC's) should be adequate. The low levels needed are a consequence of the fact that even under conditions of high disease activity the concentration of infectious agent in the blood (e.g. HIV) is many orders of magnitude lower than the concentration of RBC's.

This invention has been described by reference both to generic description and specific embodiment. Examples provided are not intended to be limiting unless so specified, and variations will occur to those of ordinary skill in the art without the exercise of inventive

faculty. The invention embraces these alternatives, save for the limitations imposed by the claims set forth below.

Claims

1. A mammalian erythrocyte bound to a first monoclonal antibody at a receptor site for which said first monoclonal antibody is specific, said first monoclonal antibody being cross-linked to a second monoclonal antibody specific for an antigen present in the mammalian primate circulatory system.

2. The erythrocyte of Claim 1, wherein said erythrocyte bears, on its surface, up to 1000 of said first monoclonal antibodies bound to said second monoclonal antibody.

3. The erythrocyte of Claim 1, wherein said erythrocyte receptor site is the CR1 protein.

4. The erythrocyte of Claim 1, wherein said antigen is selected from the group consisting of a virus, microorganism or toxic chemical.

5. The erythrocyte of Claim 1, wherein said antigen is a non-immunogenic substance found in the mammalian primate circulatory system, which can become pathogenic or cause adverse biological effects if present in sufficient amounts.

6. The erythrocyte of Claim 4, wherein said antigen is HIV (The AIDS virus).

7. The erythrocyte of Claim 5, wherein said antigen is low density lipoprotein.

8. The erythrocyte of Claim 1, wherein said erythrocyte is a human or other primate erythrocyte.

9. The erythrocyte of Claim 8, wherein said monoclonal antibodies are obtained from a non-human host, or from human sources.

10. A method of therapy treating a mammalian primate individual having an antigen present in its circulatory system, comprising inoculating said individual with a monoclonal antibody heteropolymer comprising a first monoclonal antibody specific for a receptor site on the surface of the erythrocyte of said individual, said first monoclonal antibody being cross-linked to a second monoclonal antibody specific for said antigen, said inoculation being performed in sufficient amounts to permit binding of sufficient antigen to erythrocytes via said antibodies to reduce the amount of free antigen in circulation in said individual to a level below that which is cytopathic or which causes adverse biological effects to said individual. The inoculation may be performed either directly with said heteropolymer, or indirectly by franking the patient's erythrocytes (or erythrocytes from a compatible donor) with said heteropolymer, followed by inoculation of the franked erythrocytes.

FIG. 1a

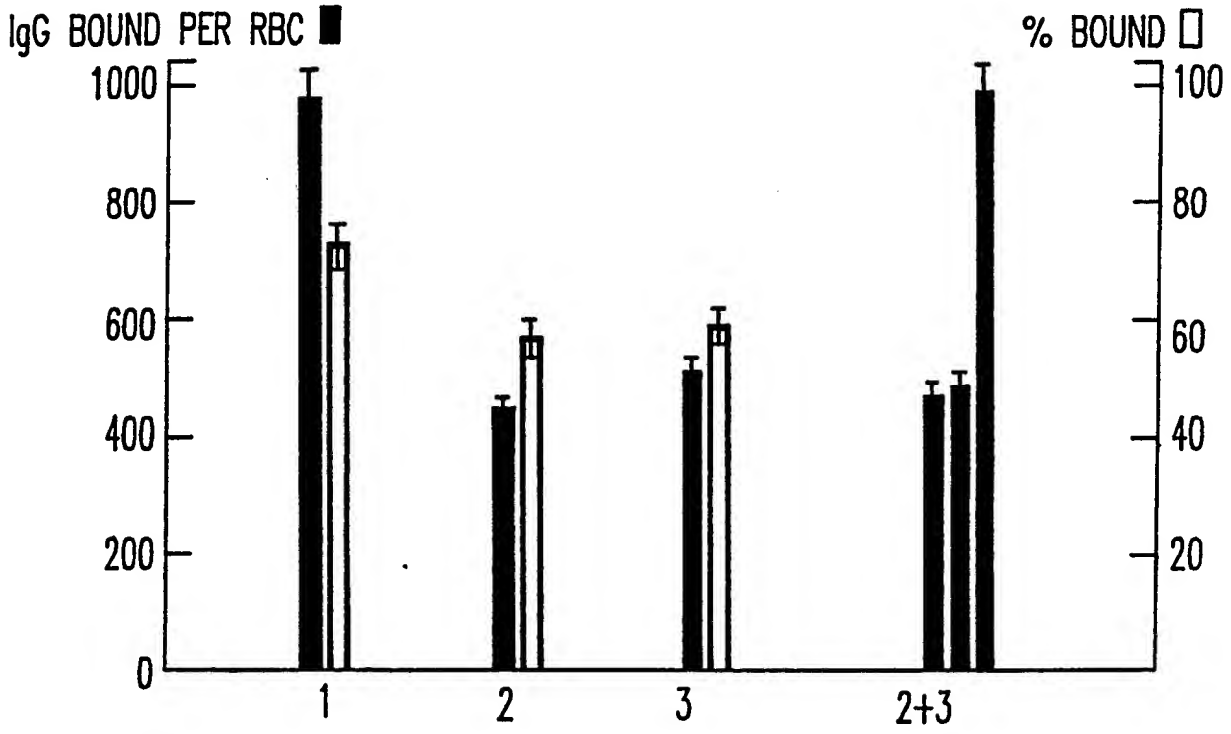
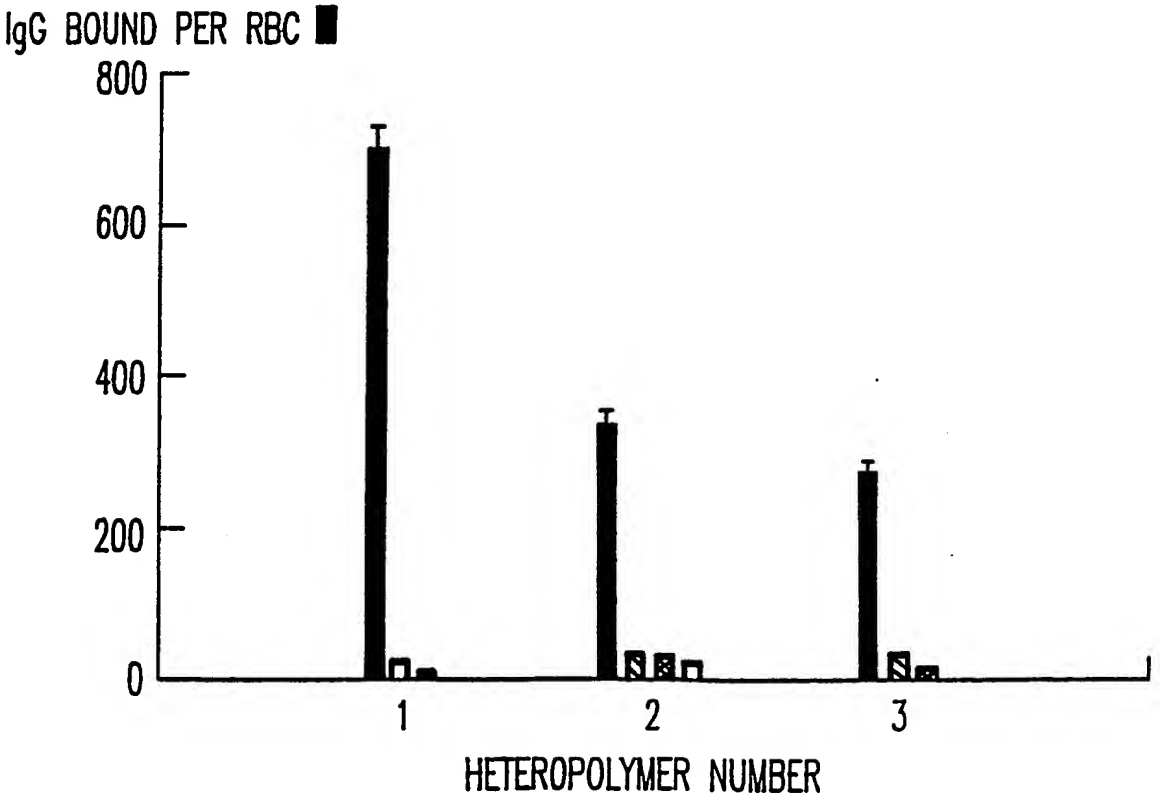

IgG BOUND PER RBC
% BOUND
1000
800
600
400
200
0
100
80
60
40
20
1
2
3
2+3

FIG. 1b


IgG BOUND PER RBC
800
600
400
200
0
1
2
3
HETEROPOLYMER NUMBER

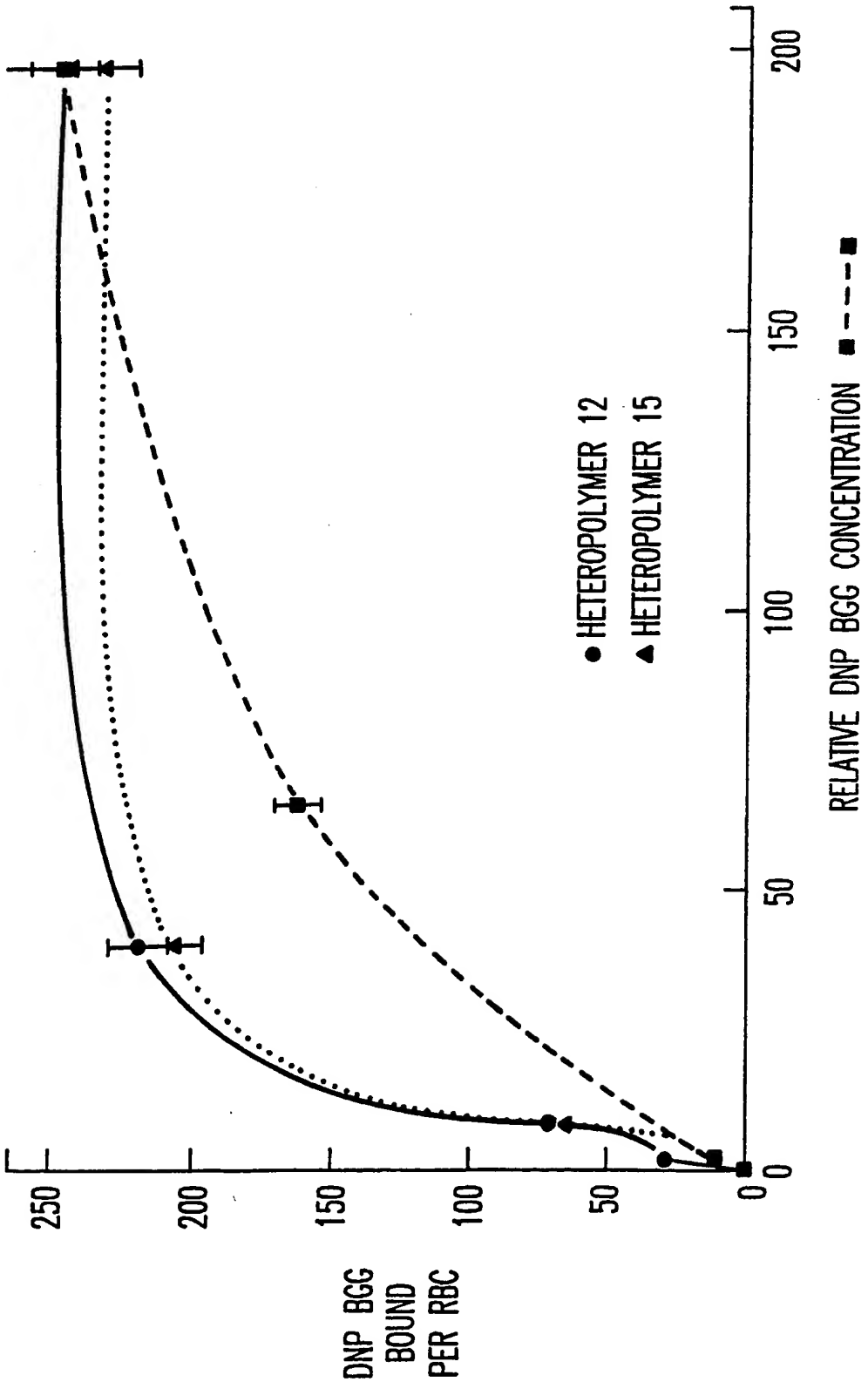

DNP BGG BOUND PER RBC
250
200
150
100
50
0
0
50
100
150
200
RELATIVE DNP BGG CONCENTRATION
● HETEROPOLYMER 12
▲ HETEROPOLYMER 15

FIG. 2

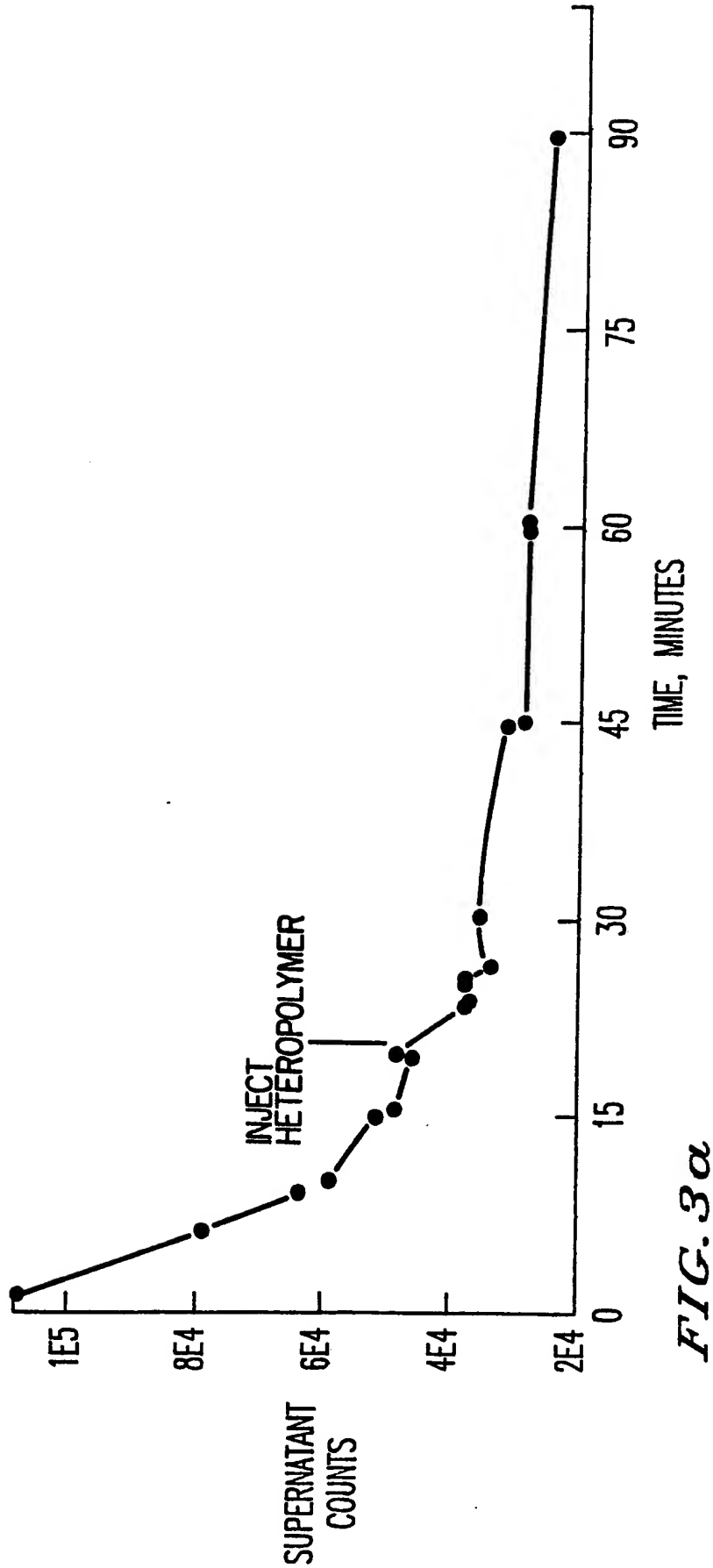

SUPERNATANT COUNTS
1E5
8E4
6E4
4E4
2E4
0
15
30
45
60
75
90
TIME, MINUTES
INJECT HETEROPOLYMER

FIG. 3a

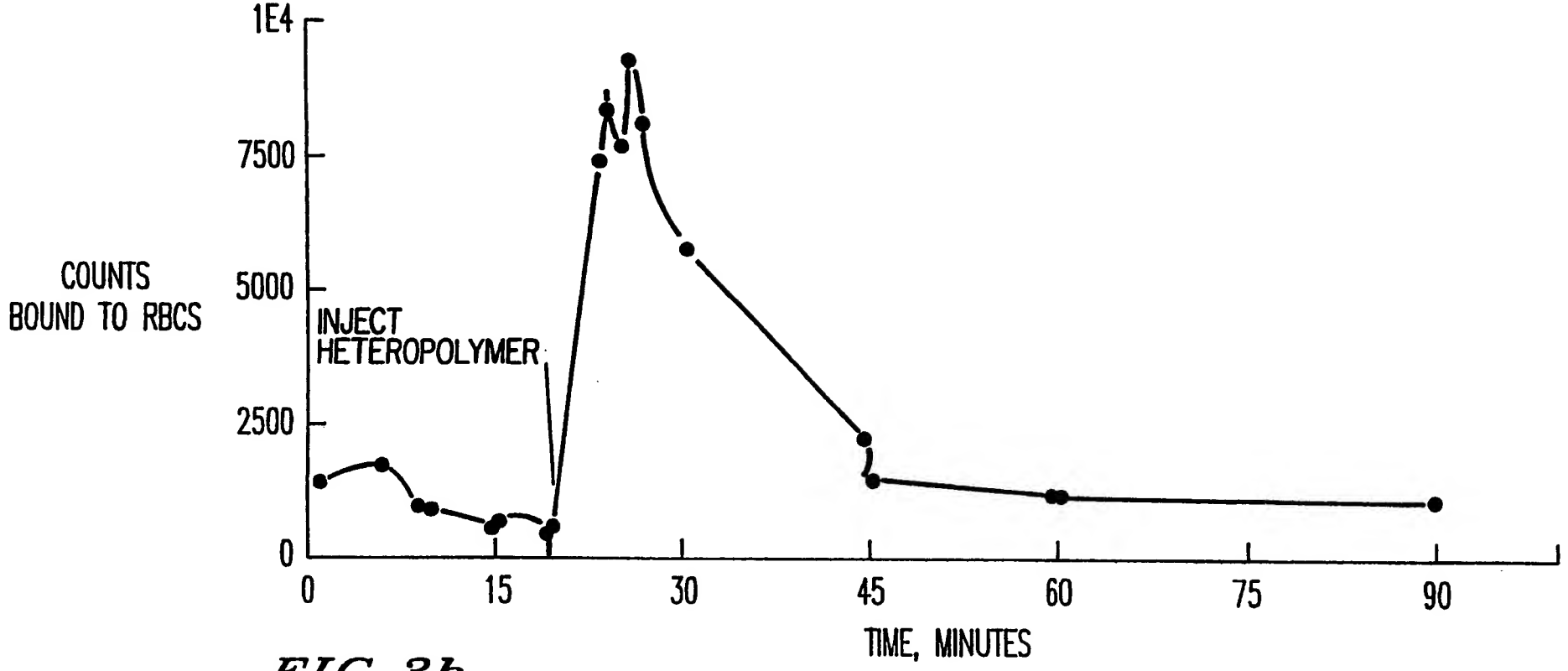

1E4
7500
5000
2500
0
COUNTS
BOUND TO RBCS
INJECT
HETEROPOLYMER
0
15
30
45
60
75
90
TIME, MINUTES

FIG. 3b

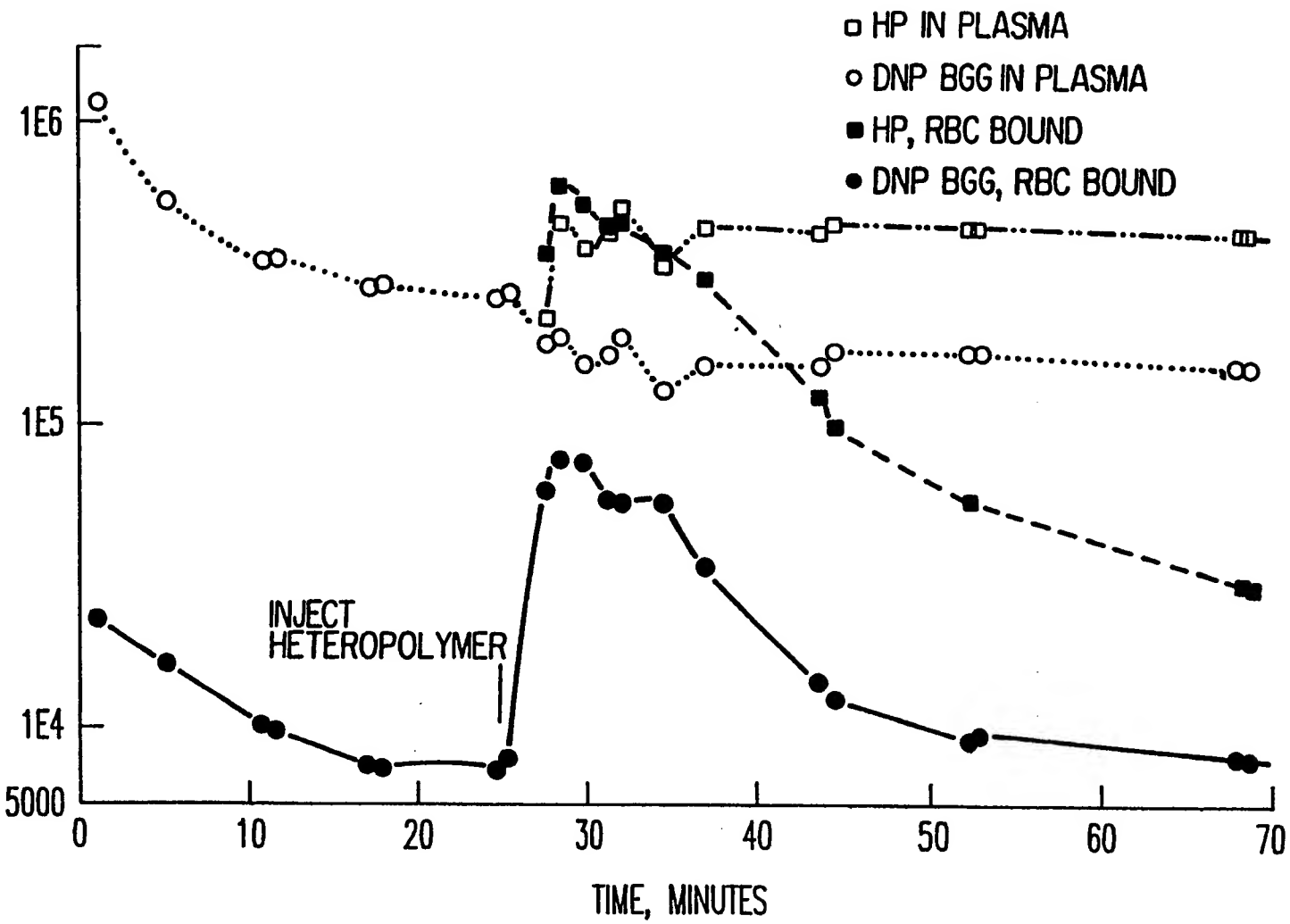

HP IN PLASMA
DNP BGG IN PLASMA
HP, RBC BOUND
DNP BGG, RBC BOUND
COUNTS, 1311-DNP BGG, OR 1251-HP
1E6
1E5
1E4
5000
INJECT HETEROPOLYMER
0
10
20
30
40
50
60
70
TIME, MINUTES

FIG. 4a

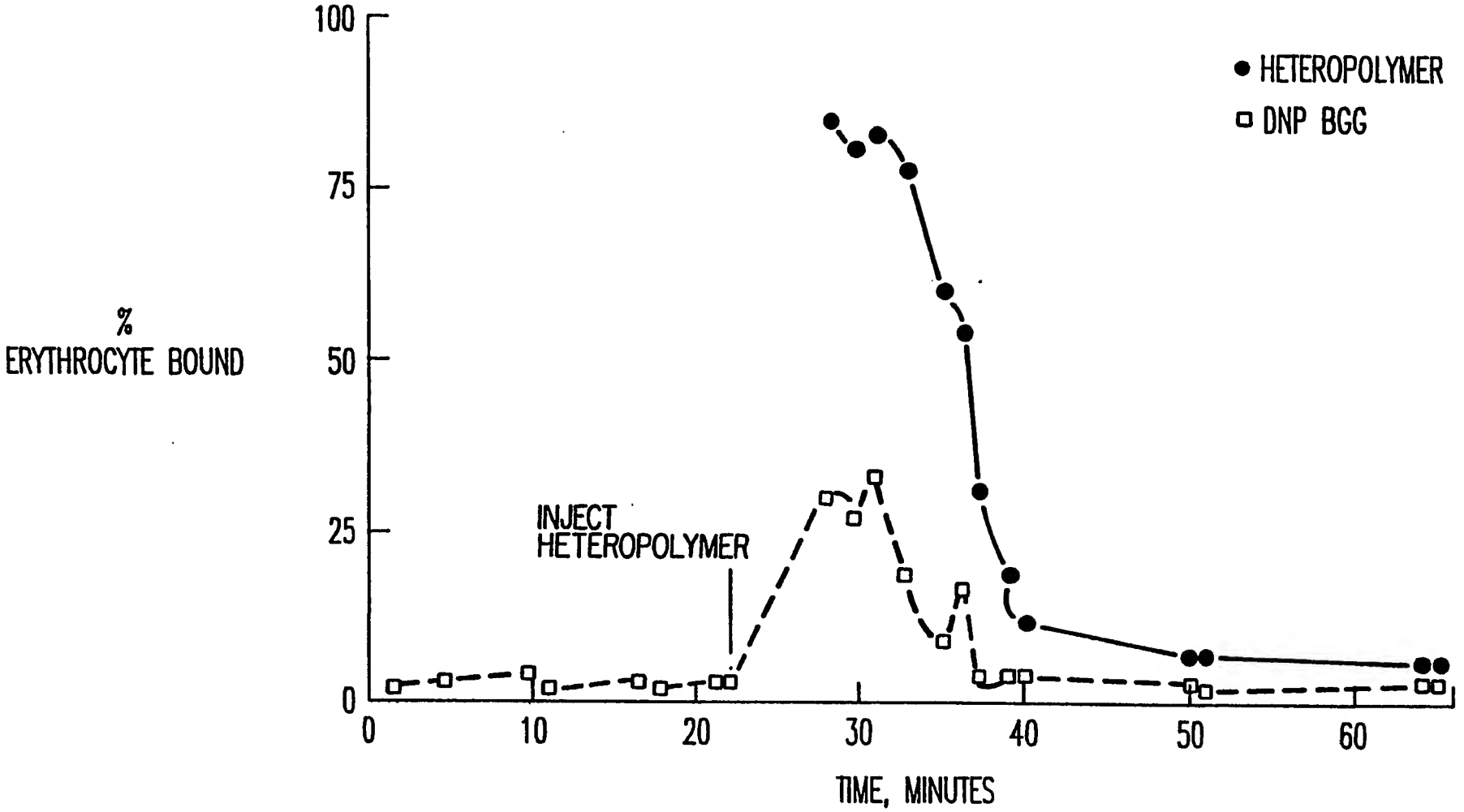

100
75
50
25
0
% ERYTHROCYTE BOUND
HETEROPOLYMER
DNP BGG
INJECT HETEROPOLYMER
0
10
20
30
40
50
60
TIME, MINUTES

FIG. 4b

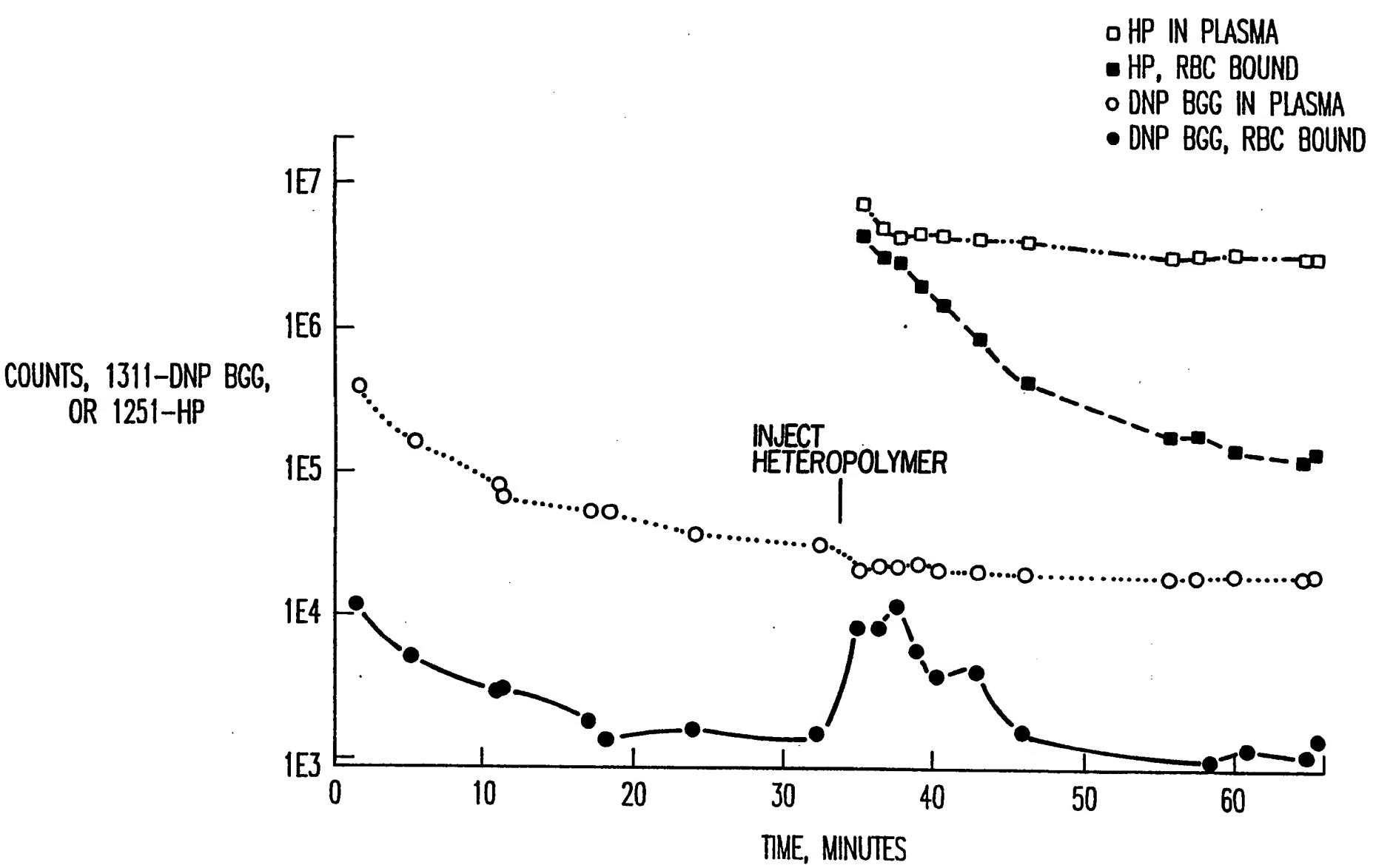

HP IN PLASMA
HP, RBC BOUND
DNP BGG IN PLASMA
DNP BGG, RBC BOUND
COUNTS, 1311-DNP BGG, OR 1251-HP
1E7
1E6
1E5
1E4
1E3
INJECT HETEROPOLYMER
0
10
20
30
40
50
60
TIME, MINUTES

FIG. 5a

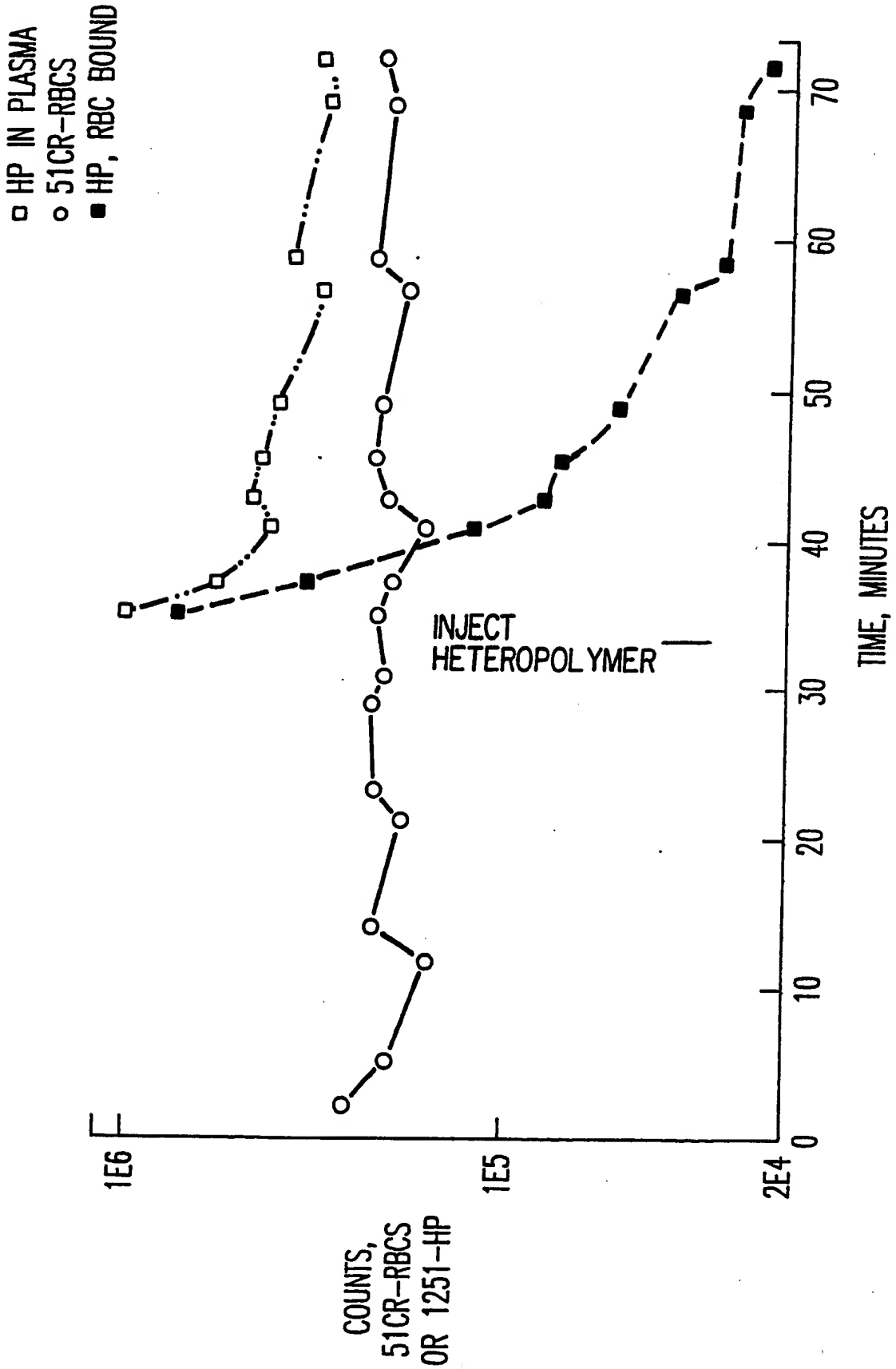

HP IN PLASMA
51CR-RBCS
HP, RBC BOUND
INJECT
HETEROPOLYMER
COUNTS,
51CR-RBCS
OR 1251-HP
1E6
1E5
2E4
0
10
20
30
40
50
60
70
TIME, MINUTES

FIG. 5b

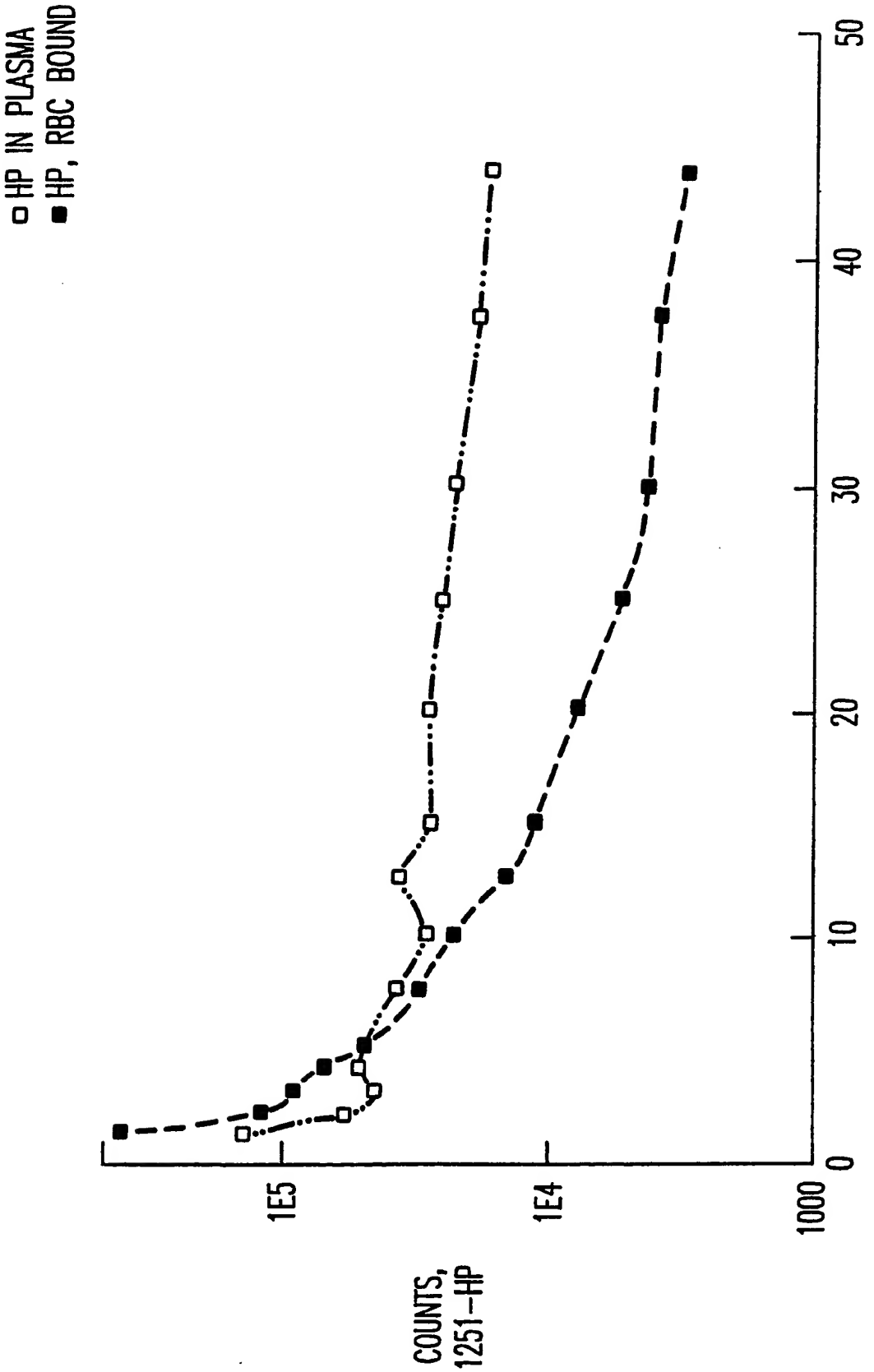

HP IN PLASMA
HP, RBC BOUND
COUNTS, 1251-HP
1E5
1E4
1000
0
10
20
30
40
50
TIME, MINUTES

FIG. 6a

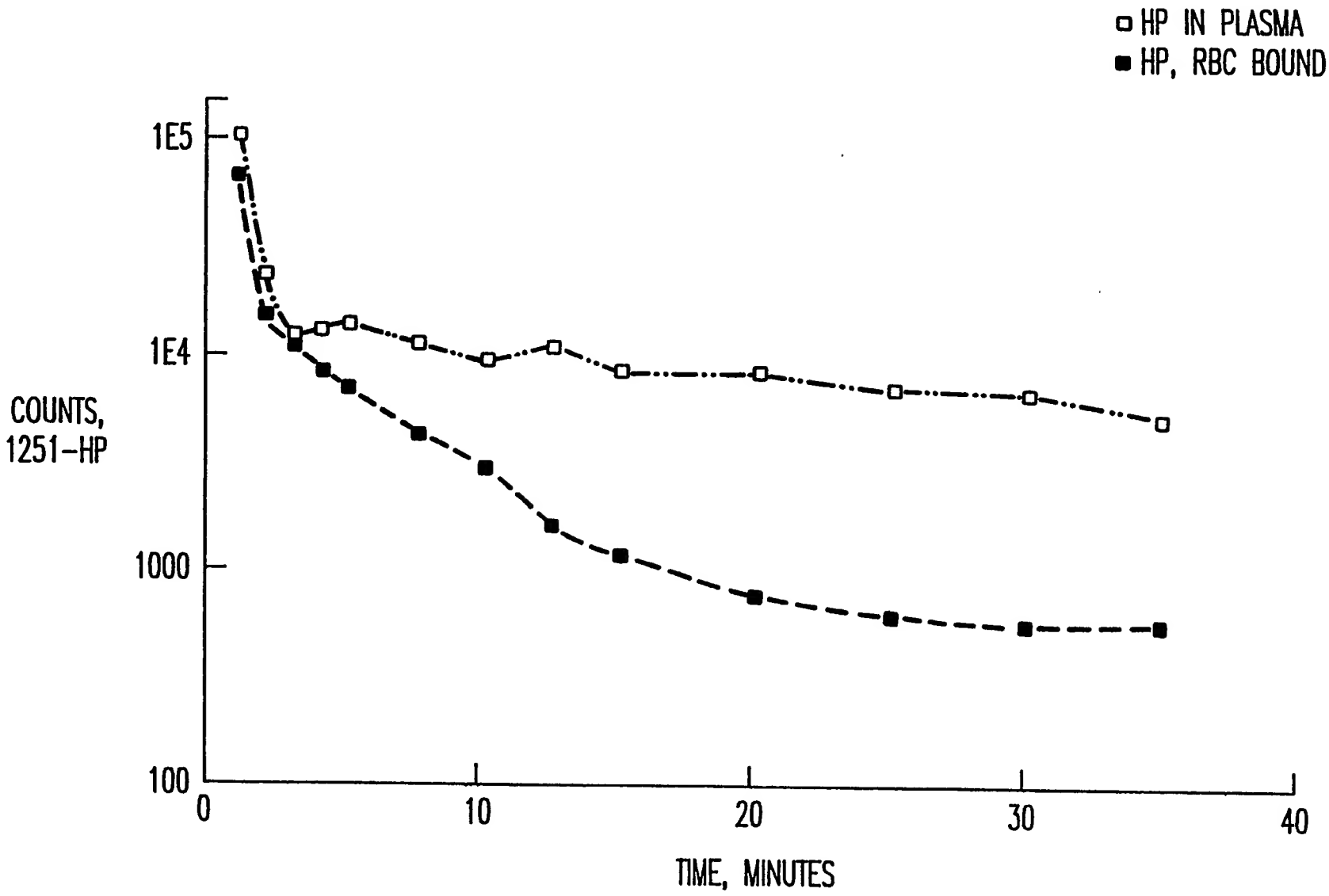

HP IN PLASMA
HP, RBC BOUND
1E5
1E4
1000
100
COUNTS,
1251-HP
0
10
20
30
40
TIME, MINUTES

FIG. 6b

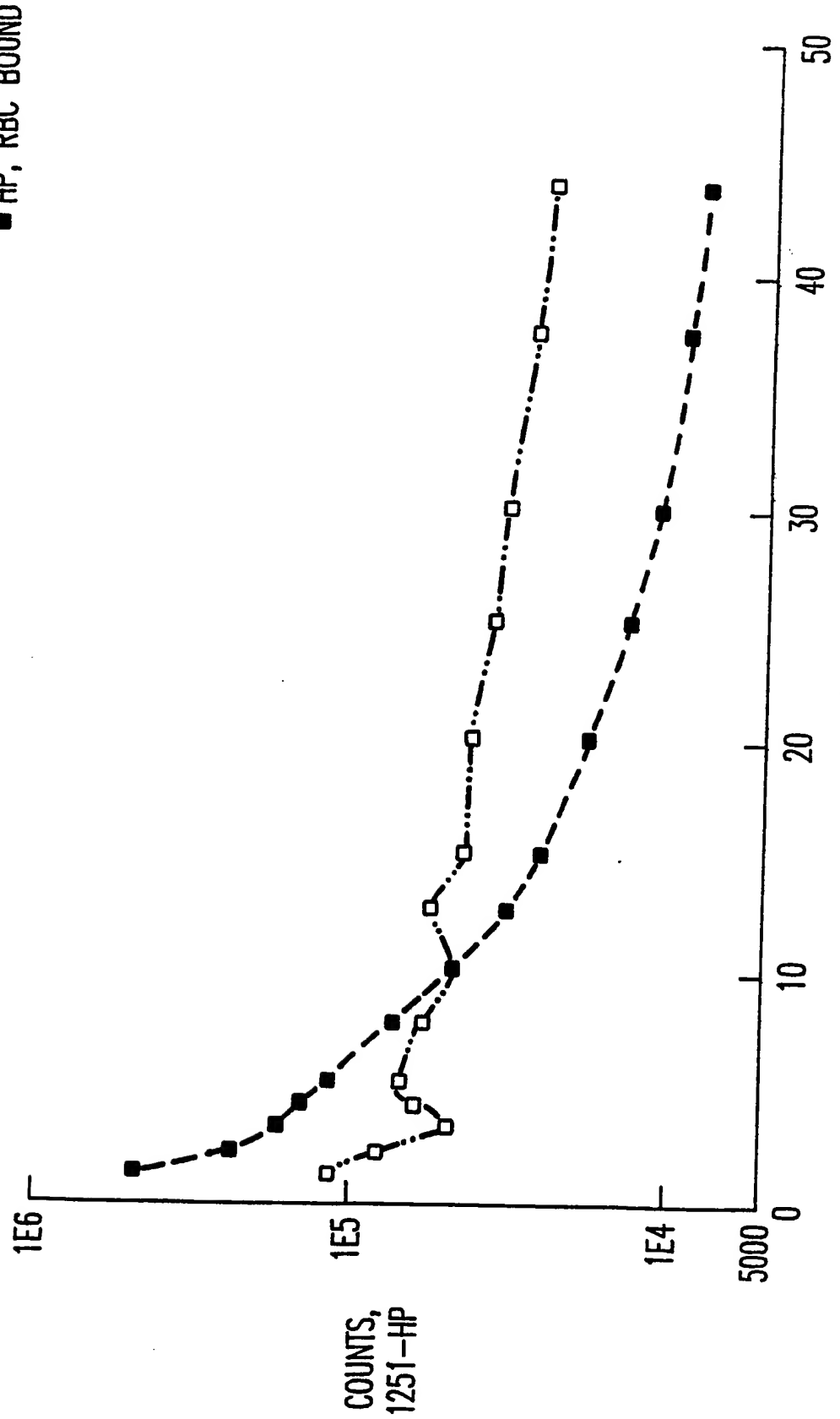

HP IN PLASMA
HP, RBC BOUND
COUNTS, 1251-HP
1E6
1E5
1E4
5000
0
10
20
30
40
50
TIME, MINUTES

FIG. 6c

INTERNATIONAL SEARCH REPORT

International Application No PCT/US91/07158

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): A61K 39/395 , 35/14

U.S. CL.: 435/2; 424/85.8; 604/4

II FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	435/2; 424/85.8; 604/4

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [9]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	Journal of Experimental Medicine. Volume 160. issued December 1984. Karpovsky et al., "Production of Target-Specific Effector Cells Using Hetero-Cross-Linked Aggregates Containing Anti-Target Cell and Anti-Fc Receptor Antibodies". pages 1686. lines 6-11 and 21-22: page 1687. lines 1-10: page 1688. lines 4-22: page 1691. lines 1-10 and figure 2: page 1697. Table V and lines 14-29.	1-10
Y	The Journal of Immunology. Volume 139. Number 9. issued 01 November 1987. Titus et al. "Human K/Natural Killer Cells Targeted With Hetero-Cross-Linked Antibodies Specifically Lyse Tumor Cells In Vitro and Prevent Tumor Growth In Vivo". pages 3153-3158. See Abstract.	1-10

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
16 January 1992	30 JAN 1992
International Searching Authority	**Signature of Authorized Officer**
ISA/US	George C. Elliott ebw

Form PCT/ISA/210 (second sheet) (Rev.11-87)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	The Journal of Immunology, Volume 139, Number 11, issued 01 December 1987, Edberg et al. "Quantitative Analysis of The Binding of Soluble Complement-Fixing Antibody/dsDNA Immune Complexes to CR1 on Human Red Cross Cells". pages 3739-3747. See entire article.	1-10